EXPLAINING RARE MENDELIAN PHENOTYPES: EXOME SEQUENCING AND FUNCTIONAL ANALYSIS OF SPONDYLOMETAPHYSEAL DYSPLASIA WITH CONE-ROD DYSTROPHY

by
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Abstract

Mendelian disorders are a set of heritable phenotypes that come at a significant cost for patients, their families, and the healthcare system. Here we describe and implement strategies to determine the genetic basis of unexplained Mendelian disorders through next-generation sequencing. Using whole-exome sequencing, we found that a novel heterozygous variant in *COL2A1* underlies Stanescu dysplasia, and that biallelic variants in *PCYT1A* cause spondylometaphyseal dysplasia with cone-rod dystrophy (SMD-CRD). In addition to detecting causal variants for phenotypes of interest, next-generation sequencing has the potential to reveal incidental findings in genes unrelated to a patient's primary phenotype. We find that incidental findings arise frequently in clinically actionable genes, yet our ability to interpret them is extremely limited, highlighting the need for improved and standardized variant classification methods. Finally, we generated and characterized various cellular models for the characterization of SMD-CRD alleles in *PCYT1A*. Our studies suggest that full or partial loss of *PCYT1A* expression has variable and cell-type specific consequences, resulting in altered lipid metabolism and lipid droplet formation, accelerated chondrocyte differentiation, and varying degrees of *PCYT1A* loss-of-function. We conclude that concerted efforts are required to fully elucidate the genetic and mechanistic bases of Mendelian phenotypes, and that sustained efforts are required for the accurate interpretation of human genetic variation detected by next-generation sequencing studies.

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Preface

I owe the Human Genetics Graduate Program tremendous thanks for investing in me and in my graduate education. In many ways, I feel as though I've learned more in these past five years than in my entire pre-graduate school life, and witnessing the fascinating advances by colleagues and friends has been a constant impetus for me to push forward with my own research. The Human Genetics Program and all its affiliated faculty and staff, particularly the members of the executive committee (Drs. David Valle, Kirby Smith, Andy McCallion, Susan Michaelis, and Roger Reeves, and Ms. Sandy Muscelli) have been constant sources of support throughout my graduate career and have made completion of my degree possible. Thanks to this program, I am certain that genetics is my calling and couldn't be more excited to shape a career in this area.

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The Valle lab has been my family way from home for the last few years, and I've been incredibly fortunate to have such wonderful colleagues and friends there throughout graduate school. Cassandra Obie and Gary Steel have been a wealth of information for all my cell culture and molecular biology experiments, as well as steadfast sources of support, advice, and laughter. My fellow graduate students in the lab, Sarah Robbins and Jing You, have been tremendously compassionate, supportive, and fun, and I've learned a great deal from both of them. Furthermore, members of the Avramopoulos, Vernon, and Sobreira labs have been like my extended lab family and have helped me in many ways; particularly I would like to thank Nicole Eckart, Xuan Pham, Ruihua Wang, Kyra Feuer,

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Chapter 1: Introduction

The utility of studying rare Mendelian disorders

Mendelian disorders are a collection of heritable phenotypes transmitted from parents to their children according to Mendelian modes of inheritance. Approximately 8% of all viable human births have a Mendelian condition that manifests by adulthood, and Mendelian disorders affect a total of 25 million Americans (Chong et al., 2015). Many of these disorders are extremely debilitating and are of high burden for patients, their families, and the healthcare system (Angelis, Tordrup, & Kanavos, 2015). Mendelian disorders range from severe, multi-systemic phenotypes such as Hutchinson-Gilford progeria syndrome to milder, more targeted phenotypes such as isolated strabismus. Mendelian disorders vary in their population frequencies, and in some instances the same disorder can vary in frequency depending on the population being studied, as pathogenic alleles may be enriched in specific populations. An example of this is Tay-Sachs disease, for which 1:300 individuals in the general population are heterozygous carriers of pathogenic alleles, in contrast to the 1:30 heterozygous carrier rate in the Ashkenazi Jewish population (MIM 272800; www.omim.org).

Because many monogenic Mendelian disorders affect only small numbers of individuals, the utility of studying them comes into question, especially when research resources are limited. As a form of justification, we can think of Mendelian phenotypes as naturally occurring model systems. They enable us to see how an isolated genetic defect can impact larger biological processes, and in this way dissect systems which would otherwise be far too complex to understand all at once. Furthermore, studying monogenic extremes sometimes provide insights which are more broadly applicable to

the population at large. An example of this is Brown and Goldstein's discovery of biallelic variants in the gene encoding the low-density lipoprotein receptor in individuals with familial hypercholesterolemia (Goldstein & Brown, 2015). By studying this severe pediatric disorder, Brown and Goldstein were not only able to dissect more generalizable mechanisms for LDL uptake and cholesterol metabolism, but also were able to implicate heterozygous LDL receptor variants in less severe cardiovascular phenotypes affecting 1:500 individuals. Finally, the basic research that stemmed from uncovering this genetic defect led to the discovery and implementation of statin drugs, which have been used to reduce LDL levels and prevent cardiovascular disease in the population at large. Thus, understanding extreme cases that occur naturally in the population can ultimately lead to broader clinical applications and help us to understand genetic variation as a whole.

Application of whole-exome and whole-genome sequencing to explain rare Mendelian disorders

Next-generation sequencing has ushered in a brave new era for diagnosis and treatment of Mendelian disorders. Previously, disease gene discovery relied on hypothesis-driven testing for disease-associated genes and pathogenic variants, and as such was biased toward existing knowledge of variation in humans and other model systems. Exome and genome sequencing enable unbiased evaluation of genetic variation in order to identify candidate causal variants, and thus enable the discovery of completely novel mechanisms for disease. Less than a decade ago, Ng et al. demonstrated that exome sequencing can be applied to uncover the genetic basis of a Mendelian disorder (S. B. Ng et al., 2010) and opened the floodgates for applying exome sequencing on a broader scale.

In 2012, the NIH, NHGRI, and NHLBI collectively initiated the Centers for Mendelian Genomics (CMGs), a collection of centers across the US dedicated to finding the genetic bases of unexplained Mendelian disorders through next-generation sequencing. One of the CMGs is the Baylor-Hopkins Center for Mendelian Genomics, led by investigators at Johns Hopkins University and Baylor University. As an initial step toward identifying all genetic variants and genes contributing to Mendelian phenotypes, the CMGs began by applying exome sequencing to understand the genetic basis of a variety of Mendelian disorders. Although the exome only comprises ~1-2% of the genome, variants in protein-coding sequences are responsible for the majority of explained Mendelian disorders to-date (Chong et al., 2015). Furthermore, exome sequencing is currently less expensive than genome sequencing, so a larger number of phenotypes can be investigated by applying this approach as an initial step. As of December 16, 2017, http://mendelian.org/mendelian-traits-numbers stated that 63% of currently known Mendelian phenotypes have a known molecular basis, and 18% of human genes have been shown to harbor variants that cause a Mendelian phenotype. Thus, we are making great headway in understanding Mendelian phenotypes and their genetic bases, but much of the exome still remains unexplained.

Hurdles for gene and variant discovery and functional characterization

Though next-generation sequencing together with analytic approaches that incorporate genetic and genomic information have solved many Mendelian disorders, several factors have prevented complete success of these studies. First, these disorders typically are rare, which prevents obtaining sufficient statistical evidence for genetic causality, and pedigrees large enough for convincing segregation analysis are difficult to

find. In some cases, unaffected or more mildly affected family members may also be mosaic for variants of interest, which confounds analyses. Second, our ability to phenotype is limited by the systems subjected to clinical examination, the expertise and thoroughness of examination, and the age of the patients being studied. In some cases, phenotypes are missed simply because clinicians don't yet know to look for them, or because they have not yet manifested in a clinically recognizable way. Both these factors confound our ability to cluster families for analytical purposes, or to perform proper segregation analyses within families for late-onset conditions.

Moreover, there is often phenotypic overlap among genetically disparate disorders (for instance, joint hypermobility in multiple forms of Ehlers-Danlos as well as other disorders). Another issue is locus heterogeneity (variants in different genes causing similar phenotypes), since the same candidate gene may be mutated in only a single family. Furthermore, allelic heterogeneity (different variants in the same gene observed in independent individuals with the same phenotype) complicates variant interpretation, as many variants fall in disease-associated genes but are not pathogenic in themselves. We will explore this concept further in Chapter 3.

Nearly a quarter of all phenotype-associated genes have been described in connection with more than one Mendelian phenotype, which further confounds our ability to interpret variants (December 16, 2017, http://mendelian.org/mendelian-traits-numbers). I will provide an example of this in Chapter 5 through the case of biallelic variants in *PCYT1A* causing three distinct or partially overlapping phenotypes. A separate problem is that some disorders appear to follow classic Mendelian patterns of inheritance, but are not monogenic; this can also contribute to reduced penetrance and

variable expressivity, which pose additional challenges for segregation analyses. Furthermore, genes may require environmental interactions in order for protection against disease or to spur disease manifestation (e.g. fava bean consumption leading to hemolytic anemia in association with G6PD deficiency; MIM 305900).

Another hurdle is obtaining strong evidence for variant causality, particularly given a large list of candidate variants. For instance, in conducting exome sequencing of an affected parent and child with an autosomal dominant disorder, we may find ~100 rare (minor allele frequency < 0.01), functional (coding or splice site) variants shared between both affected individuals. I will discuss such a case in Chapter 2. Some genes such as *TTN* also have large coding sequences, and as such are larger targets for mutation. This makes it extremely difficult to interpret variation that arises in these genes. In some instances, certain variants may also be in linkage disequilibrium with truly pathogenic variants, which can lead to improper flagging of variants as causal. Finally, strong candidate variants may be identified but lack additional supporting functional evidence to implicate them in disease pathogenesis. This issue can be compounded by inaccessibility of relevant tissue types for certain disorders. Finally, although exome sequencing enriches for protein-coding regions, it excludes putatively functional noncoding elements such as promoters or enhancers, as well as much of the 3' and 5' untranslated regions and introns. Our ability to detect copy number variants as well as structural variants from exomes is also limited. In combination, all these factors have barred us from having a more complete understanding of Mendelian disorders and their genetic bases. However, they have also provided lessons for conducting future analyses more effectively and innovatively.

Finally, once candidate variants have been identified, detailed functional studies must be conducted in order to trace the path from a genetic defect to its corresponding disease mechanism. Although this process may be straightforward, biological systems are often more complex than we anticipate. The consequences of genetic variants may be tissue- or cell-type-specific, and disease mechanisms may not be overtly connected to the known functions of candidate genes, as we will see in Chapter 5. Though riddled with challenges, increasingly sophisticated methods for functional studies are necessary for truly understanding Mendelian disorders and treating them to improve patient outcomes. Next-generation sequencing studies have invigorated a whole new cycle of functional interrogation and biological understanding, which will only continue to grow as we move forward.

Chapter 2: Novel *COL2A1* variant (c.619G>A, p.Gly207Arg) manifesting as a phenotype similar to progressive pseudorheumatoid dysplasia and spondyloepiphyseal dysplasia, Stanescu type

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Julie Jurgens's Contributions: Exome sequence analysis and identification of *COL2A1* as primary candidate gene for this family's condition; Sanger validation of variant and segregation analysis; identification of second family with the same *COL2A1* variant and skeletal dysplasia phenotype at an ASHG annual meeting; coordination of efforts between clinicians and researchers in U.S. and Korea for phenotypic comparison of probands, correspondence, and ongoing collaboration; conceptualization and drafting of manuscript and figures; coordination of revisions from other co-authors; coordination of submission process; drafting of response to reviewer comments and integration of additional responses from other co-authors; manuscript finalization with journal for publication; generation of graphical abstract as requested by journal

Introduction

Progressive pseudorheumatoid dysplasia (PPRD; MIM 208230), is a painful, disabling autosomal recessive skeletal dysplasia accompanied by pseudorheumatoid arthritis that manifests in childhood and inexorably worsens. Features include platyspondyly, epimetaphyseal expansion, and progressive stiffness and swelling of the joints (Garcia Segarra et al., 2012). Hurvitz et al. (1999) implicated variants in *WISP3* (Wnt-1-inducible secreted protein 3; MIM 603400) in several families with PPRD; however, no *WISP3*

variants were found in a simplex case in this cohort (Family 6), suggesting locus heterogeneity (Hurvitz et al., 1999).

Spondyloepiphyseal dysplasia (SED), Stanescu type, is distinguished by painful, progressive joint space narrowing, joint contractures, platyspondyly, and epimetaphyseal dysplasia with pronounced femoral coxa valga and normal stature. This disorder was first described by Stanescu et al. (1984) in a male proband born of unaffected parents, making the mode of inheritance indeterminate. Subsequently, Nishimura et al. (1998) described two families with multiple affected generations and autosomal dominant transmission (Nishimura et al., 1998). As noted by Nishimura et al. (1998), SED, Stanescu type is likely underdiagnosed, perhaps due to shared features with other dysplasias. Neither the genetic basis of this disorder nor is its relationship to PPRD is clear.

Czech dysplasia (MIM 609162) is caused by a heterozygous c.823C>T, p.Arg275Cys variant in the type II alpha-1 collagen gene *COL2A1* (MIM 120140). While also characterized by early-onset, progressive arthritis and skeletal dysplasia, it is clinically distinguishable from PPRD by shortened metatarsals in all described individuals (Marik, Marikova, Zemkova, Kuklik, & Kozlowski, 2004).

Here we describe three patients from two families harboring a novel heterozygous variant in *COL2A1* (RefSeq NM_001844.4: c.619G>A, p.Gly207Arg), with similar phenotypes.

Clinical Description

The proband of Family 1 (Table 1) was previously reported as the simplex case of family 6 by Hurvitz et al. (1999). She is the product of unaffected, non-consanguineous parents with negative family histories for relevant features. As a child, she was noted

never to hop or run. A waddling gait was initially assessed at 4 years of age, prompting concerns of limb weakness, but extensive evaluation demonstrated no neuromuscular abnormality. Joint pain began around 5 years of age. More severe joint pain and early joint immobility arose in late childhood and were initially most severe in her neck and spine.

Although radiographs at 9 years of age showed mild platyspondyly, anterior vertebral wedging and mild, generalized epiphyseal abnormalities, she carried a diagnosis of juvenile rheumatoid arthritis throughout childhood. She developed kyphoscoliosis in adolescence and had extension osteotomies of both femora at age 13 years due to worsening hip flexion contractures.

She was first evaluated through the Midwest Regional Bone Dysplasia Clinics at age 22 years (Fig. 1a-c). Between childhood and her first assessment, joint pain progressively worsened, with greatest severity in her elbows, hips, knees and hands. Progressive joint stiffness and joint prominence developed, particularly in her hands. She had an adult height of ~150 cm, generalized truncal stiffness, severe neck stiffness, normal craniofacial features, and limited mouth opening. Shoulder abduction and elevation as well as elbow extension, pronation and supination were limited. Legs showed hip flexion contractures, only 15° of rotation, no abduction or adduction, minimal active hip flexion, mild knee flexion contractures, and ankle stiffness. Her stance was a "Z" posture (Fig. 1a-b) with absent heel strike, knee flexion, and hip flexion. Spine radiographs showed platyspondyly and anterior wedging (Fig. 1c).

Over the ensuing ten years, her stiffness and pain worsened, requiring bilateral hip replacements at age 25 years and knee replacements at age 26 years. She developed

progressive thoracolumbar kyphosis and presented acutely at age 35 years with progressive numbness to the level of the umbilicus and leg weakness. MRI showed severe spinal stenosis and multilevel myelomalacia. She had laminectomy (T8-L1 and L4-L5) and spinal fusion, which improved her symptoms. Today, she is 38 years old and remains ambulatory.

She was initially diagnosed with PPRD and included in the cohort of Hurvitz et al. (1999), but had no pathogenic *WISP3* variants, which was confirmed by subsequent testing (Supp. Materials and Methods). A diagnosis of Czech dysplasia was also considered but rejected due to the absence of structural abnormalities of the toes or metatarsals.

Concerns arose in the proband's daughter (Table 1) during childhood. She was born at 36 weeks gestation with a weight of 2840 g and length of 48 cm. When first assessed at 3 $^{7}/_{12}$ years of age, her mother was concerned about a mild waddling gait, but examination was normal. By 5 years of age, she had progressive knee valgus, internal tibial torsion, mild limitation of ankle dorsiflexion, and poor heel-toe transition, but was otherwise normal. Between 7 and 8 ½ years of age, neck stiffness, minimal limitation of elbow supination, and minimal wrist stiffness were evident, as were confounding features caused by an unrelated subtrochanteric femur fracture.

By age 11 ½ years, she had developed worsening neck stiffness; pain in her neck, hips, and knees; increased waddling; and decreased walking endurance. She had decreased mobility in her neck, elbows, wrists, hips, knees, and ankles. Proximal interphalangeal hand joints were prominent, and feet were normal except for incidental mild hallux valgus. Height was 135.4 cm (5th centile). Between 12-15 years of age,

kyphosis developed, mild scoliosis progressed, and joint stiffness and pain worsened, particularly in her hips. She is now a young adult and attends college. Joint immobility, particularly of the hips and knees, has continued to progress. She has severe pain with ambulation and can only walk for short distances with crutches. Both hip replacement and spinal fusion surgeries are anticipated.

Radiographs at 5 and 8 years of age were normal. By 12 years of age, her spine showed mild to moderate generalized platyspondyly, mild anterior wedging, loss of anterior substance and mild excess concavity of the vertebral bodies, and a lumbar scoliosis (Fig. 1d). There was mild, generalized joint space narrowing and metaphyseal irregularity (distal radii, distal ulnae, and distal femora; Fig. 1e-g), and phalangeal metaphyses of the hands were mildly prominent (Fig. 1g). Femoral heads were flattened and irregular, and femoral necks were short (Fig. 1e). The left femur was deformed secondary to previous injury.

The proband of Family 2 (Table 1) is a Korean boy who presented at age 6 years because of awkward gait and running difficulty. He was the product of a normal full-term pregnancy of unaffected, non-consanguineous parents and born with a weight of 3.7 kg. Motor and cognitive developments were normal, and he started to walk alone at 14 months of age. His parents noticed his slow, waddling gait and inability to run at 2-3 years of age. He was examined by a pediatric neurologist but not found to have neurologic or muscle disease. His height was 118.3 cm (z=+0.6, 73th centile), and weight 18.4 kg. His older brother and sister were unaffected. At age 8 $^{1}/_{2}$ years, he had an awkward gait and complained of difficulty squatting. He could run only slowly, and could barely climb stairs without the railing. Physical assessment revealed standing

posture with hips and knees flexed. He showed a wide-based gait with smooth heel-toe progression. The knees showed 20° flexion contractures. The hips and elbows showed mild limitation of motion. His height was 131.5 cm (z=+0.45, 67th centile) and arm span was 137 cm.

At 9 $^{4}/_{12}$ years of age, his knee and hip joint pain worsened, precluding sports activities. He had a stiff gait with hip abduction. The hip joints showed 15° flexion contractures, and further flexion was painful and limited to 100°. Nonsteroidal anti-inflammatories and diverse physical treatments lessened his joint pain. By 10 $^{1}/_{2}$ years of age, he could walk only with assistance due to joint pain and limb muscle weakness from prolonged disuse. Bearing weight on his legs resulted in hip, knee, and ankle pain. Motion at the hip joint was painful, with flexion contractures of 20° and flexion limited to 120°. The knee joint showed 20° flexion contractures.

His initial hip radiographs at 4 years of age (Fig. 1h) showed irregular contour of the femoral capital epiphyses, shallow acetabulum, and wide femoral neck with mild coxa valga. Skeletal survey at age 8 $^{1}/_{2}$ years (Fig. 1i-m) revealed generalized platyspondyly with wedged vertebral bodies at the thoracolumbar junction. His pelvis showed mild capital femoral epiphyseal dysplasia, coxa valga, and small, elongated ilia. Hand radiographs showed no distinct metaphyseal widening of the phalanges or metacarpals. Feet lateral radiographs showed large os trigonum bilaterally, and accentuated pes cavus. We prioritized a diagnosis of SED, Stanescu type and also included a broad category of unclassified spondyloepiphyseal dysplasias, possibly including a type II collagenopathy. Based on the proband's average to tall stature,

apparent spondyloepiphyseal dysplasia with coxa valga, and absent metaphyseal widening of the phalanges, there was no initial suspicion of PPRD.

Results

After multiple assessments failed to detect *WISP3* variants in Family 1, we performed WES on the proband, her unaffected mother, and her affected daughter. WES analysis and filtering in Family 1 (Supp. Materials and Methods) yielded 56 heterozygous variants, including a novel *COL2A1* variant (p.Gly207Arg). Sanger sequencing confirmed that this variant was present in the 2 affected individuals and absent in the unaffected mother of the proband (Supp. Figure S1), but we were unable to confirm *de novo* occurrence in the proband since paternal DNA was unavailable. In the proband of Family 2, we directly evaluated *COL2A1* for sequence and copy number variants because we suspected a type II collagenopathy (Supp. Materials and Methods). We detected the same heterozygous variant in the proband by full Sanger sequencing of *COL2A1* (Supp. Table S1) and confirmed *de novo* occurrence by Sanger sequencing of the mutation site in his unaffected parents (Supp. Figure S2). Functional prediction software indicates that the variant occurs at a conserved residue and is predicted to be damaging (Supp. Materials and Methods). This variant was submitted to a *COL2A1* locus-specific database at http://databases.lovd.nl/shared/variants/COL2A1, DB-ID COL2A1_000405, in three separate entries representing each individual with the variant.

Discussion

Interestingly, the clinical and radiological findings manifest somewhat differently between affected individuals in Families 1 and 2, although they share the same variant. The proband of Family 2 exhibited normal height until late childhood, and the proximal femora were broad and elongated with coxa valga. The proband of Family 2 also lacked enlarged phalangeal epimetaphyses of the hands, which is one of the hallmarks of PPRD and was prominent in Family 1.

COL2A1 encodes the alpha-1 chain of type II collagen, the primary collagen in articular cartilage. Many bone dysplasias are type II collagenopathies (MIM 120140), ranging from lethal to very mild and including Czech dysplasia. Although the individuals described here share many phenotypic features and the autosomal dominant mode of inheritance of Czech dysplasia, they lack major hallmarks including metatarsal shortening, which is thought to be uniformly characteristic of Czech dysplasia (Table 1). In addition, a single heterozygous *COL2A1* variant (p.Arg275Cys) has been ascribed to Czech dysplasia in all published cases (MIM 609162), while the individuals described here are heterozygous for a p.Gly207Arg variant.

Although there are few reports of Stanescu dysplasia, all describe a chondrocytic phenotype of PAS-positive, amylase-resistant cytoplasmic inclusions (Stanescu et al., 1984; Nishimura et al., 1998). Since we did not have access to chondrocytes from our patients, we were unable to test for this feature. However, given the phenotypic overlap with our cases, it is likely that Stanescu dysplasia is also a type II collagenopathy, and conceivable that it arises from the same mutation described here. We believe it would be

worthwhile to investigate the possibility of *COL2A1* mutation in other cases with this phenotype.

Rukavina et al. (2014) implicated a c.611G>T, p.Gly204Val *COL2A1* variant in a case of early-onset progressive osteoarthritis with mild spondyloepiphyseal dysplasia (Table 1; Rukavina et al., 2014). This patient shares many clinical features of the patients described here, suggesting similar consequences of the p.Gly204Val and p.Gly207Arg *COL2A1* variants.

Previous connections have been made between WISP3 and type II collagen. Overexpression of wild-type *WISP3*, but not PPRD variants, increases type II collagen in human chondrocytes (Sen, Cheng, Goldring, Lotz, & Carson, 2004; M. Wang et al., 2013). WISP3 normally co-localizes with type II collagen in the midzone region of chondrocytes, but WISP3 localization is disrupted by PPRD variants (Sen et al., 2004). Type II collagen secretion into the extracellular matrix also decreases in human chondrocytes transfected with mutant as compared to wild-type *WISP3* (M. Wang et al., 2013) We hypothesize that the *WISP3* variants found in PPRD and the *COL2A1* variant described here both decrease type II collagen, potentially disrupting the extracellular matrix and common downstream signaling pathways (Barber, Esteban-Pretel, Marín, & Timoneda, 2014).

These individuals heterozygous for the p.Gly207Arg *COL2A1* variant have a phenotype overlapping PPRD, SED, Stanescu type, and Czech dysplasia, although the third is clinically distinguishable by metatarsal shortening. The individuals described here have a disorder that further expands the phenotypic spectrum of type II collagenopathies.

We initially matched the common variant in these families at an academic conference. The team working with the proband of Family 2 described the variant in an abstract, which was subsequently recognized by the group following Family 1. While this story highlights the power of case matching, the probability that such matches will be made is enhanced by tools such as GeneMatcher (Sobreira, Schiettecatte, Boehm, Valle, & Hamosh, 2015). Based on our experience, we strongly support wider collaboration and data sharing, particularly for researchers and clinicians following rare disorders.

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We are grateful to our patients and their families for participation and to Dr. James Hyland of Connective Tissue Gene Tests for molecular analysis of *WISP3* in Family 1. Our work was supported in part by grants from the US NIH/NHGRI (T32GM07814; 1U54HG006542) and the Genome Technology to Business Translation Program of the National Research Foundation funded by the Ministry of Science, ICT & Future Planning of the Korean Government (NRF-2014M3C9A2064684).

Chapter 2 Figures

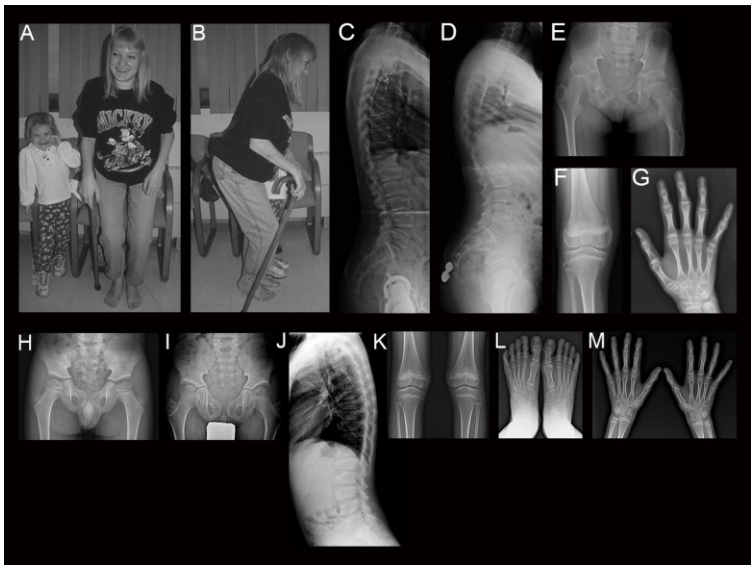


Figure 1. Clinical features of patients. a-c: Proband of Family 1 at 23 years. Note marked joint contractures resulting in Z posture. **c:** Lateral spine radiograph shows platyspondyly and irregular degenerative changes. **d-g**: Radiographs of the daughter of the proband of Family 1 at 12 years. **d:** Mild anterior wedging and platyspondyly of lateral spine. **e:** Hip and pelvic features include flattened and irregular femoral heads, short femoral necks, and deformed shaft of the left femur secondary to prior trauma. **f:** Knee radiograph shows joint space narrowing and metaphyseal irregularity. **g:** Note metaphyseal prominence and joint space narrowing at distal radius and distal ulna. **h-m:** Radiographs of the proband of Family 2 at 4 years (**h**) and 8 years (**i-m**). **h:** Note irregularity and mild flattening at articular surfaces of capital femoral epiphyses. Acetabulum is flat, and femoral necks are broad with mild coxa valga. **i:** Capital femoral epiphyses show mild flattening and dysplastic trabeculation. Femoral necks are broad and elongated with further coxa valga. Ilia are somewhat narrowed and elongated. The

acetabulum shows deepening with irregularity of its contour. **j:** Note moderate spinal platyspondyly with anterior wedging and irregularity of the endplates. **k:** Knee joints show mild flaring of the distal femora and proximal tibiae. Epiphyses are normal. **l-m:** Hands and feet are normal.

Chapter 2 Tables: Table 1. Comparison of Clinical and Molecular Features of Relevant Disorders

	Family 1	**Family 2**	**Rukavina et al. [2014]**	**Czech Dysplasia**	**SED, Stanescu type**	**Progressive Pseudorheumatoid Dysplasia (PPRD)**
Mode of Inheritance	Autosomal dominant	*De novo*	Autosomal dominant	Autosomal dominant	Autosomal dominant	Autosomal recessive
Family Origin	U.S.A.	Korea	Unknown	Multiethnic	Multiethnic	Multiethnic
Major Clinical Features						
Short Stature	+	-	+	-	-	+
3rd and 4th Toe Shortening	-	-	-	+*	-**	-
Hearing Loss	-	-	-	+/-	+/-***	-
Joint Pain	+	+	+	+	+	+
Progressive Joint Immobility	+	+	+	+	+	+
Joint Replacement Surgery Needed	+	Not yet	+	+	+	+
Major Radiologic Features						
Progressive Platyspondyly	+	+	+	+	+	+
Progressive Joint Space Narrowing	+	?	+	+	+	+
Metaphyseal Enlargement, Hands	+	?	+	+/-	+	+
Metatarsal Shortening	-	-	-	+*	-	-
Mutated Gene	*COL2A1*	*COL2A1*	*COL2A1*	*COL2A1*	Unknown	*WISP3*
Genetic Variant****	c.619G>A (heterozygous)	c.619G>A (heterozygous)	c.611G>T (heterozygous)	c.823C>T (heterozygous)	N/A	Allelic Heterogeneity [Hurvitz et al., 1999; Delague et al., 2005; Zhou et al., 2007; Yue et al., 2009; Temiz et al., 2011; Dalal et al., 2012; Garcia Segarra et al., 2012; Sun et al., 2012]
Protein Variant****	p.Gly207Arg (heterozygous)	p.Gly207Arg (heterozygous)	p.Gly204Val (heterozygous)	p.Arg275Cys (heterozygous)	N/A	Allelic Heterogeneity (See references above)
SIFT/ PolyPhen-2 Scores	0/1	0/1	0/0.999	0/0.999	N/A	N/A

*Although the patients described by Burrage et al. [2013] did not have disproportionate shortening of the 3rd and 4th toes, they did have radiologic evidence of generalized shortening of the metatarsals.

**One proband described by Nishimura et al. [1998] had shortening of his 2nd and 3rd toes and 2nd fingers; this is distinct from the 3rd and 4th toe shortening observed in Czech dysplasia.

***The original proband described by Stanescu et al. (1984) had sensorineural hearing loss.

**** *COL2A1* variant positions are based on RefSeq transcript NM_001844.4.

Chapter 2 Supplement

Variant Detection in Family 1

WISP3 was initially investigated in the proband of Family 1 by Hurvitz et al. (1999). Subsequent clinical molecular analysis (courtesy of James Hyland, Connective Tissue Gene Tests) failed to demonstrate pathogenic *WISP3* variants in either the proband or her daughter. All five exons of *WISP3* were PCR-amplified and sequenced using the ABI 3730 sequencer, and *WISP3* was analyzed for copy number variants using a CTGT high-density targeted array. Both these methods failed to detect any pathogenic sequence or copy number variants in *WISP3*.

Next, we performed whole exome sequencing on the proband, her unaffected mother, and her affected daughter at the Baylor-Hopkins Center for Mendelian Genomics. The patients were counseled on the possible outcomes of whole exome sequencing and signed a consent form approved by the Johns Hopkins University School of Medicine Institutional Review Board. To capture the CCDS coding regions and adjacent intronic sequences, we used the Agilent SureSelect Human All Exon V4 51Mb capture kit to obtain paired-end 100 bp reads on the Illumina HiSeq2500 platform. Reads were aligned to the reference genome (NCBI human genome assembly build 37; Ensembl core database release 50_361 (Hubbard et al., 2009)) using the Burrows-Wheeler Alignment (BWA) tool (H. Li & Durbin, 2009), and single nucleotide variants (SNVs) and small insertion-deletions (indels) were identified using SAMtools (H. Li et al., 2009). We performed local realignment and base call quality recalibration using GATK2.3.9 (DePristo et al., 2011; McKenna et al., 2010). We then selected for variants with a minimum of 8 reads, root mean square mapping quality of 25, strand bias p-value below

10^{-4}, and end distance bias below 10^{-4}. Using a presumed autosomal dominant inheritance model, we prioritized variants using the Analysis Tool of PhenoDB (Hamosh et al., 2013). We selected for rare heterozygous variants with MAF<0.01 in the 1000 Genomes Project (Build 20130502; 1000 Genomes Project Consortium et al., 2012); dbSNP builds 126, 129, and 131 (Sherry et al., 2001); Exome Variant Server (release ESP6500SI-V2); and our in-house control database (CIDRVar 51Mb), and nonsynonymous exonic or splicing SNVs and indels which were present in affected individuals but absent in unaffected individual.

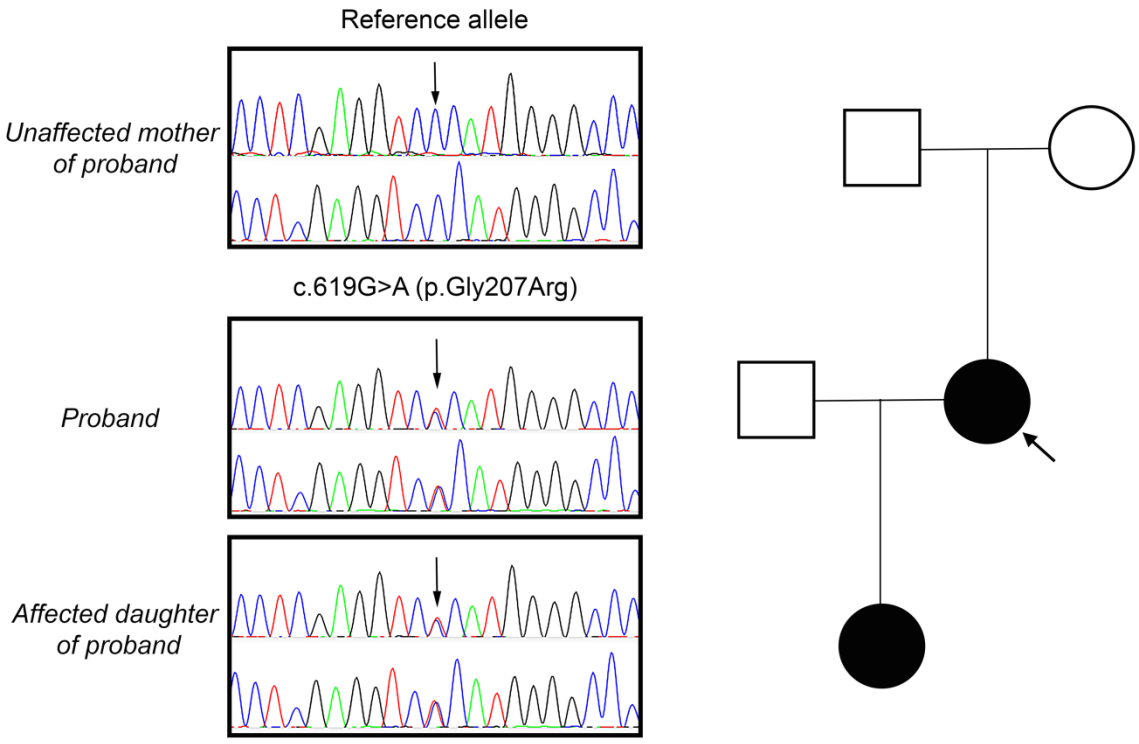
Variant Detection in Family 2

We directly sequenced the *COL2A1* gene in the proband of Family 2 because his radiological findings suggested a spondyloepiphyseal dysplasia. Genomic DNA was extracted from peripheral blood leukocytes. The *COL2A1* gene was screened by direct Sanger sequencing of all coding exons and their flanking regions (Supp. Table S1). Multiplex ligation-dependent probe amplification (MLPA) analysis was performed to screen gross gene deletions and duplications involving this gene using the SALSA P214 *COL2A1* (MRC-Holland, Amsterdam, Netherlands). Upon detection of the variant of interest, Sanger sequencing of the mutation site was performed on both unaffected parents of the proband to confirm co-segregation of the variant with the phenotype (Supp. Figure S2).

Prediction of Variant Pathogenicity

The novel *COL2A1* variant (RefSeq transcript NM_001844.4: c.619G>A, p.Gly207Arg) was assessed for putative conservation and functionality using a variety of established methods. The variant has a GERP++ rejected substitutions score (Genomic

Evolutionary Rate Profiling score, GERP++ version) of 4.81 (Davydov et al., 2010); a SIFT score of 0 (SIFT v. 1.03, score range 0-1.0, with scores ≤0.05 predicted damaging; Kumar, Henikoff, & Ng, 2009); and a PolyPhen-2 score of 1 (score range 0 (benign) to 1.0 (probably damaging), PolyPhen-2 v.2.2.2; Adzhubei et al., 2010). These scores indicate that the variant occurs at an evolutionarily conserved residue and is predicted to be damaging.

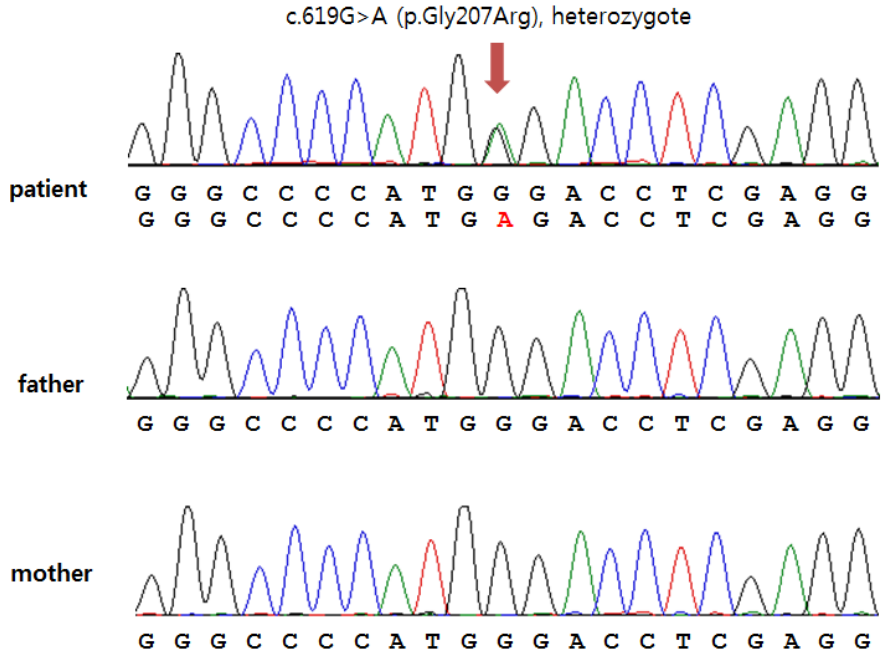


Supp. Figure 1. Sanger validation of *COL2A1* variant in Family 1. Sanger sequencing was performed to validate co-segregation of the *COL2A1* variant c.619G>A

(p.Gly207Arg, RefSeq transcript NM_001844.4) with the phenotype in Family 1. Whole exome sequencing originally detected this variant in the proband and her affected daughter, but not in her unaffected mother. Primers were designed for amplification and subsequent Sanger sequencing of a 357 bp fragment encompassing the *COL2A1* variant. Results confirm co-segregation of the *COL2A1* variant with the phenotype in Family 1 (the variant is present in the proband and her affected daughter, but absent in her unaffected mother). Primer sequences were as follows:

Forward CACCAGGATTGCCTTGAAAT

Reverse TAGAGTGTTGCGTGGGGAGT



Supp. Figure 2. Sequence analysis of *COL2A1* exon 9 in Family 2. The proband was heterozygous for the missense variant c.619G>A (p.Gly207Arg, RefSeq transcript NM_001844.4). This missense variant was not detected in his unaffected parents, indicating that it arose *de novo* in the proband.

Supp. Table 1. Primers used for Sanger sequencing of *COL2A1* gene in the proband of Family 2.

Exon	Forward primer sequence	Reverse primer sequence
1	AGCGTGACTCCCAGAGAGG	GGTGAACTTCTGCGTGTCCT
2	CCCAGCCTACATTCTTCAGC	GTGGCCTTTCCTTTCTACCC
3-6	AGAGGGGTTCAGTGGAGTCA	AGGTGACCAGGCCCTTAAAC
7	AAAAGCAATGCTGCTTGACC	GCCCTTAGCACCACAGTCTC
8	ACTTTCCAGGCATCAGTGGT	CTGGACAGCAGCCATGTTTA
9-10	CTGTCCAGGAGAAAGCAAGG	GGGGGCTGTTAGGACAGAGT
11	CCACCAGGGACTCTGTCCTA	ACTTGCCAGCACAGTCACAG
12	GGTGAGGACAGAGGAGCTTG	TCCCTCATGAGAGCCTGAGT
13-14	AGGGGTGGTCTGGGTTTTAC	CAGGATGTAGGGCTGGTGTT
15	GCATTGTCCCTCACAGGACT	ACAAGCACACCTTGCTCCTT
16	TCTGAGGCATCCTGGCTAGT	CACACTGGGGGTGAATTCTT
17	AGCCAGACTCCGTCTCAAAA	CCTCCCCCTTTCCAGTAGAC
18	CTGAAGCCAGGCAAAGTTTC	ACTGCGAGTGTCTGGCTTTT
19	TGGGTGCATGTGCATAATTT	GGCATAGGTGCTGTCCATTT
20-21	ATGTCCCTGAAATGGACAGC	AAGAGAGGCAGGTCCTCACA
22	CTCTTTCCCATGCTCTCCTG	AGGGAAGTGAGAGGGGCTAA
23	CAGTTGCCAAGGCTACTTCC	CATGTCAGTCTGGTGGTTGG
24-26	ACAGCTACTGCTCCCCAGAA	GCCTACCATCTACCCCCTGT
27	AAGCTCAGCGGTGTCTTGTT	CTTCTGTTCAGGGGCCATAA
28	TTATGGCCCCTGAACAGAAG	TGACCAATGGCAAACAGAAG
29	GTACCGTGGAGGTCTGGAAA	CTTCCTGCACCACTCAGACA
30-31	TGTCTGAGTGGTGCAGGAAG	GACATGGGTCCAGGACATTC
32-33	AGCCTCCACAGATGACACAA	GACAGCACCTCTCCCTGAAC
34	TGCATCCTGTAGGGGTCTTC	TTCATCACCAGGTGCCATAA
35-36	ACATCCTTTCCCCTGTTCCT	ACAAGACAGAACCGCCTTTG
37	ATGGGCTGCACAGTAACACA	CTCTCCTCCCACCCTAGACC
38-39	CCATCATGTGTTCAGGGTGA	CTACCAACATGGGGGTGTTC
40	CACAAGTCTCCTGTGGCAGA	TCCACAGTCAGCACTTCAGG
41	GTTCAGCTCCCTGAAGTGCT	GGTCCCCATGATCAGTTAGC
42	AGGTAGAGAAGCCCCCAGAG	TGAGAGAGGAGAGGCAGGAG
43-44	TCTGAGCTCACAGAGCATGG	CAAGTTTCCCTCCTCCTTCC
45-46	GAAGGAGGAGGGAAACTTGG	CGTCAGAGAAAAGCCAGGAC
47-48	TCAGTAAAAGCCGCCTTCC	TGGGGGAACAGCTTTATGTC

49	GACAGCTTGGGATCACCCTA	AGCGAGAGGCTGATTCATGT
50	ATGAATCAGCCTCTCGCTGT	CAGGCTGATGCCCTAAAAGA
51	CTGACTCCTTCCCTGCTGTC	CCCAGCTCTGCCCTGTACTA
52	CCTCATCCCTTGTCCCTGTA	CAGCAGGGAGCTAGTTGGAC
53	GCAGAGATGGCTCCTCAAAC	TCATGCCTCTGATGATCCTG
54	ACAGGTTGGGAGCTCACTGT	TCTGCCCAGTTCAGGTCTCT

Chapter 3. Assessment of incidental findings in 232 whole exome sequences from the Baylor-Hopkins Center for Mendelian Genomics

Chapter 3 is reprinted from the final accepted journal article:

Jurgens J, Ling H, Hetrick K, Pugh E, Schiettecatte F, Doheny K, Hamosh A, Avramopoulos D, Valle D, Sobreira N. (2015). Assessment of incidental findings in 232 whole exome sequences from the Baylor-Hopkins Center for Mendelian Genomics. *Genet Med*. 17(10): 782-8.

Julie Jurgens's Contributions: Analysis of incidental findings in 232 exome sequences; conceptualizations of portions of incidental findings analysis (with Dr. Nara Sobreira); conceptualization and drafting of manuscript and figures; coordination of revisions from other co-authors; coordination of submission process; drafting of response to reviewer comments and integration of additional responses from other co-authors; manuscript finalization with journal for publication

Introduction

Incidental findings in whole exome and whole genome sequencing are variants of known or possible pathology identified in genes unrelated to the initial reason for which sequencing tests were ordered. As large-scale sequencing is increasingly used in a clinical setting, standardizing procedures for identifying, classifying, and reporting variants is of great importance. In 2013, the American College of Medical Genetics and Genomics (ACMG) released a recommendation suggesting that known pathogenic or expected pathogenic constitutional variants detected in 56 genes representing 24 conditions with well-known guidelines for prevention or treatment should be reported to patients sequenced in a clinical setting (Green et al., 2013). Shortly after the release of

these recommendations, the Clinical Sequencing Exploratory Research (CSER) Consortium and Electronic Medical Records and Genomics (eMERGE) Network followed by releasing a set of guidelines for the return of incidental findings within genomics research studies (Jarvik et al., 2014). These guidelines recommended that medically actionable incidental variants be returned to willing research participants, but exempted researchers from an obligation to search for these variants.

Although incidental variants are unexpected, when variant pathogenicity is certain, the information enables patients to make proactive decisions about their health and inform their physician. However, some variants are of uncertain significance, or initial interpretation of variant pathogenicity may be subject to change (Bell et al., 2011). Reporting these variants of uncertain significance (VUS) to patients may lead to misinterpretation of the results, produce unnecessary anxiety, and diminish confidence in genetic test results in general. Thus, transmittal of these results requires accurate and thoughtful genetic counseling and careful evaluation of available sources of variant classification to maximize benefits and minimize harm to patients.

At the Baylor-Hopkins Center for Mendelian Genomics (BHCMG, http://mendeliangenomics.org), we utilize whole exome sequencing to detect variants responsible for Mendelian disorders with unknown molecular bases. Since we sequence across the exome, we not only capture variants that are responsible for the patient phenotype, but also incidental variants which may predispose and/or cause other unrelated disorders. Returning meaningful results to research participants through their physicians is a goal of our center, so understanding the clinical significance of incidental

findings detected at the research level and knowing which of these should be returned to patients is of special interest.

Although the ACMG guidelines for reporting incidental findings are geared toward a clinical setting, they provide a well-defined, targeted set of genes from among the many interrogated in research sequencing. Thus, our results on this set may inform policies for handling incidental findings. Here we examine the implementation of the ACMG clinical guidelines within a research setting by assessing the spectrum of rare, functional variation in the 56 genes in whole exome sequences of 232 individuals in 89 families sequenced at the Baylor-Hopkins Center for Mendelian Genomics for a variety of Mendelian disorders with unknown molecular bases, some of which have features that overlap with the disorders covered by the ACMG list. We then assessed variant interpretation by evaluating classification and reportability according to available databases and approaches taken by others in the literature.

Materials and Methods

All research participants were counseled on the possible outcomes of whole exome sequencing and signed a consent form approved by the Johns Hopkins University School of Medicine Institutional Review Board. Participants chose whether they would like to receive information regarding primary or incidental variants found in the course of this study and were given the opportunity to opt out or in at any time.

Whole exome sequences from 232 individuals in 89 families sequenced at Johns Hopkins University (JHU) were analyzed for rare splicing or nonsynonymous SNVs and

indels in the 56 reportable genes from the ACMG guidelines using the analysis feature of the web-based system PhenoDB (Hamosh et al., 2013).

Briefly, whole exome sequencing was done on the Illumina HiSeq2000 platform (Illumina, Inc., San Diego, CA) using paired-end 75 or 100 bp reads. 51 Mb of genomic DNA comprised of CCDS exons and adjacent intron sequences were captured with Agilent SureSelect Human All Exon V4 51Mb Kit (Agilent Technologies, Santa Clara, CA). FASTQ files were aligned to the reference genome (GRCh37; Ensembl core database release 50_361; Hubbard et al., 2009) with the Burrows-Wheeler Alignment (BWA 0.5.10) tool (H. Li & Durbin, 2009) resulting in SAM/BAM output (H. Li et al., 2009). PCR duplicates were flagged with Picard (http://picard.sourceforge.net). Local realignment around indels, base call quality score recalibration and reduced reads BAMs were performed with GATK 2.3-9 (McKenna et al., 2010). Multi-sample SNV and indel calling was performed with GATK's UnifiedGenotyper on the reduced reads BAM files. Variant sites were filtered with GATK's Variant Quality Score Recalibration best practices (DePristo et al., 2011), and heterozygous genotypes were excluded if they did not have at least 5 alternate allele reads. Sequencing coverage of the 56 genes is listed in Supplementary Table S1.

We selected for nonsynonymous SNVs, exonic indels, and splice junction variants (2 most 5' and 3' intronic bases) in each of the 56 reportable genes. Nonsynonymous SNVs and exonic variants were defined by RefSeq Gene coordinates (Pruitt, Tatusova, & Maglott, 2004). Next, we filtered these variants on the basis of their minor allele frequencies (MAF), with exclusion of variants with MAF $\geq$ 0.01 in the 1000 Genomes Project (April 2012 release; 1000 Genomes Project Consortium et al., 2012), Exome

Variant Server (http://evs.gs.washington.edu/EVS/), dbSNP build 137 (Sherry et al., 2001), and our in-house control database (CIDRVar 51Mb), which includes data from 50 individuals sequenced in-house with the Agilent51Mb exome capture kit (Agilent Technologies, Santa Clara, CA).

Next, we analyzed the variants for prior classification in the Human Gene Mutation Database (HGMD; Stenson et al., 2003), ClinVar (Landrum et al., 2014), and the *Emory* Genetics Laboratory *Variant* Classification Catalog (Bean, Tinker, da Silva, & Hegde, 2013). We then defined the burden of variants in each individual sequenced and the mutability of each gene (number of rare functional variants/gene and number of rare functional variants/kb of coding region), and the classification and reportability of each variant.

Results

In the 232 exomes, we identified 249 distinct variants in the 56 gene ACMG target set, some of which were seen in more than one individual, for a total of 391 variants. A total of 124 variants were shared between family members, and 18 variants were shared between unrelated individuals. There was an average of 1.69 variants per individual, with a range of 0-7 variants per individual. There were only 45 people (19.4%) with no variants, and in the 232 exomes we found at least one variant in 45 of the 56 genes (80.4%; Supplementary Table S1).

We then stratified these 249 distinct variants by type (Table 1). Most variants were missense (231/249, or 92.8%). There were also 4 nonsynonymous/splice variants

(1.6%), 2 splice variants (0.8%), 3 frameshifting indels (1.2%), 5 nonframeshifting indels (2.0%), and 4 nonsense variants (1.6%).

Next, we checked the classification of these variants in three databases: HGMD, ClinVar, and Emory. Of the 249 distinct variants identified, 126 (50.6%) were classified by at least one of the three databases: 54/126 (42.8%) were represented solely in HGMD; 44/126 (34.9%) were in HGMD and ClinVar; 23/126 (18.3%) were in ClinVar alone; 2/126 (1.6%) were in Emory alone; and another 3/126 (2.4%) were in all three databases (Figure 1).

In total, 101/249 variants (40.5%) were listed in HGMD (94 missense SNVs (93.1%), 2 nonsynonymous exonic/splicing variants (2.0%), 2 splicing variants (2.0%), 1 nonsense SNV (1.0%), and 2 nonframeshifting indels (2.0%)). More importantly, HGMD classified 74 (72.8%) as disease-causing mutations (DM), 24 (24.3%) as possible disease-causing mutations (DM?), 1 (1.0%) as a functional polymorphism (FP), and 2 (1.9%) as disease-associated polymorphisms (DP) reported to be in significant association with disease ($p<0.05$) along with some evidence of functionality.

Of the 148 variants not classified by HGMD, 23 variants in 13 genes were described by ClinVar and 2 variants in 2 genes were listed in Emory. This left a total of 123/249 variants (49.4%) that were not present in any database (Figure 1).

The classification of variants within these three databases was often discordant. Of the 101 variants represented in HGMD, 48 were also in ClinVar. Three of these 48 variants were also in Emory. Almost half of these shared variants (22/48 variants, or 45.8%) were given discordant classifications among databases (Supplementary Table S2).

Next, we examined the effects of gene size and evolutionary constraint on the number of functional variants within each gene, in a manner similar to that described by Petrovski et al. (Petrovski, Wang, Heinzen, Allen, & Goldstein, 2013). Some genes consistently had greater variation than others. For instance, *BRCA2*, *APOB*, *CACNA1S*, and *DSP* had the greatest number of variants/gene (Supplementary Table S1). Since increased coding length increases the target size for mutation, larger genes are expected to have a higher number of variants than smaller genes. To explore this possibility, we analyzed the number of variants per gene against the size (kb) of the consensus coding sequence (CCDS) of the gene. We found that the number of variants per gene generally increased with the size of coding sequence, and the number of variants showed a 62.8% correlation with the size of the consensus coding sequence (Figure 2).

We also found that some genes (e.g. *RB1*, *PMS2*, and *MLH1*) consistently have a lower variant density (variants/kb coding sequence) than others (e.g. *MYBPC3*, *TMEM43*, and *MYL3*) (Supplementary Table S1). The values of variant density range from 0.4 variants per kb in *RB1* to 3.4 variants per kb in *MYL3*. As described by others, a possible explanation for this variation in mutational burden despite correction for coding sequence length is varying degrees of evolutionary constraint (Petrovski et al., 2013). Certain genes are under a higher degree of purifying selection than others, and this is likely reflected by less variation per unit coding length.

Determining Reportable Variants

To determine which variants should be reported to our research participants, we adopted a stringent set of measures outlined in a recent paper (Dorschner et al., 2013). Their criteria incorporates various lines of evidence in assessing variant pathogenicity,

including comparison between variant minor allele frequencies and incidences of the corresponding disorders; familial segregation data; observation in unrelated affected individuals; *de novo* events in a trio; and protein truncation in disorders caused by haploinsufficiency. If a variant is classified as pathogenic or likely pathogenic by these criteria, it is considered reportable back to the patient.

We applied these criteria to all 391 variants in the 56 ACMG genes detected in 232 exomes. In total, we found 2 pathogenic variants (in *MSH2* and *MYLK*) and 3 likely pathogenic variants (in *LMNA, MYBPC3,* and *MUTYH*), as shown in Table 2. One of these genes, *MUTYH*, is responsible for autosomal recessive conditions (*MYH*-associated polyposis; adenomas, multiple colorectal, *FAP* type 2; colorectal adenomatous polyposis, autosomal recessive, with pilomatricomas; MIM 608456 and 132600). Since current ACMG guidelines recommend reporting only individuals with homozygous variants in *MUTYH* (Green et al., 2013), we would not report this heterozygous variant to the patient. Since the patients in our cohort underwent whole exome sequencing to explain a variety of potential Mendelian disorders, some of which have overlapping features with the disorders covered by the ACMG list, we recognized that our cohort may have an enrichment of reportable variants as compared to the general population. Indeed, two patients, one with a likely pathogenic variant in *LMNA* and one with a likely pathogenic variant in *MYBPC3,* had clinical phenotypes which included dilated cardiomyopathy, so we did not count these two cases toward our final number of incidental variants. In total, this means that 2/232 individuals, or 0.86% of our sample, had a reportable incidental variant in one of the 56 ACMG genes.

Discussion

In June of 2014, Jarvik et al. released recommendations for dealing with incidental variants in the research arena. The authors suggested that researchers be exempted from an obligation to search for variants outside the intended scope of their study, but that actionable variants be reported to research participants when discovered (Jarvik et al., 2014). The present study describes the workflow for identification and classification of incidental findings in the Johns Hopkins component of the Baylor-Hopkins Center for Mendelian Genomics based on the ACMG recommendations and on the classification criteria from Dorschner et al. (Dorschner et al., 2013).

In 232 exomes, we identified a total of 391 rare (MAF < 1%) variants in the 56 ACMG genes (1.69 variants per individual), 249 of which were distinct variants. Most of these variants were missense (231/249, or 92.8%), and half were not classified by any of the three databases (HGMD, ClinVar, and Emory) that we used to assess variant classification (123/249 variants, or 49.4%). Since these novel variants were not represented in the variant databases, they could not be utilized for comparative analyses of classifications among the three databases.

Next we used the criteria described by Dorschner et al. to classify the 391 total variants identified in 232 exomes and found that 2/232 individuals, or 0.86% of our sample, had an incidental reportable variant in one of the 56 ACMG genes. Overall, this analysis was quite time-consuming, but we were able to automate parts of it such as filtering variants whose frequency was higher than that predicted by the frequency of their associated disorders. This step reduced the number of distinct variants from 249 (obtained in our initial analyses using a 1% MAF cutoff) to 163, so only 65.5% of the

original variants required further assessment. We were also able to automate selection of variants in the HGMD, ClinVar, and Emory classification databases using our web-based system, PhenoDB (Hamosh et al., 2013). Although we did not consider the classifications from these databases in support of variant pathogenicity or reportability, the databases provided useful citations from the literature and clinical observations for some variants. This information reduced the time required for manual review. Protein truncation was automatically predicted, but required manual inspection to confirm that protein truncation is an established cause of the disorder associated with the respective gene. The most time-consuming part of the process was reviewing the literature for each variant to count familial segregations, *de novo* events, and singleton reports. This step required additional expertise regarding diagnoses of the disorders on the ACMG list. Moreover, evaluation of cases in the literature was somewhat subjective.

Several other studies have analyzed reportable incidental findings within whole-exome or whole-genome sequences to-date. The study by Dorschner et al., whose criteria we adopted for our analysis of reportable variants, found that 14/1000 individuals sequenced (1.4%) had a pathogenic or likely pathogenic reportable variant in one of the 52 ACMG genes responsible for adult-onset conditions (Dorschner et al., 2013). Johnston et al. found that 8/572 (1.39%) individuals sequenced through the ClinSeq project had a reportable variant in one of 37 cancer genes; 23 of these are on the ACMG list (Johnston et al., 2012). These numbers are similar to ours and to those in the original ACMG guidelines, which anticipated that 1% of individuals sequenced would have a reportable variant in one of these 56 genes (Green et al., 2013).

Another recent report by Lawrence et al. detected 27/543 individuals (~5%) sequenced through the NIH Undiagnosed Diseases Program with an incidental variant in one of the 56 ACMG genes (Lawrence et al., 2014). Their method differed from ours in a few ways which may have contributed to the discrepancy in frequency of incidental findings. For instance, they used reported functional studies as evidence of pathogenicity; required fewer informative meioses to count familial co-segregation events with disease; did not apply minor allele frequency cutoffs; and used designations in variant classification databases as evidence in support of variant pathogenicity. In addition, a few individuals in their cohort had a phenotype related to the reported incidental finding, and some incidental variants were repeated due to familial transmission (Li Wang et al., 2010). These differences in methodology may have contributed to the discrepancies in numbers of reportable incidental findings.

During this process we recognized that identifying and classifying the multitude of incidental variants that arise from next-generation sequencing is a time-consuming and subjective process dependent on the particular methodologies of each individual investigator. We confirmed this last observation when we found that almost half (22/48, or 45.8%) of the 101 variants represented in at least 2 databases were given discordant classifications between them. We compared these database classifications to our variant classifications from the Dorschner et al. method (Dorschner et al., 2013) and found that variant classifications from the ClinVar and Emory databases, but not the HGMD database, were generally consistent with our variant classifications (Supplemental Table S2). This is likely because HGMD aims to provide a comprehensive listing of all reported variants and draws its classifications from reports in the literature (Stenson et al.,

2003) that may not be accurate, consistent, up-to-date, or sufficient to provide support of variant pathogenicity. For these reasons, HGMD is generally less conservative than the other databases.

The abundance of rare and novel missense variants in our cohort made accurate assessment of variant pathogenicity more challenging, since all novel and many rare missense variants must initially be classified as variants of unknown significance (VUS). For many rare variants, familial segregation data or *de novo* events in a trio were not available or sufficient to define variants as pathogenic or likely pathogenic.

When we observed *de novo* variants in a trio or co-segregation of a variant with disease, family information could be used as evidence supporting pathogenicity and reportability. Although failure of a variant to segregate might be considered evidence against pathogenicity, we did not use this information in our pathogenicity evaluations, since many disorders on the ACMG list are adult-onset and could be late to manifest in unaffected family members. Family sequence data is also useful for determining the phase of multiple heterozygous variants in a single gene, and thus can be used for finding compound heterozygous variants. When individuals rather than families are sequenced, it may be more difficult to classify variants as pathogenic or likely pathogenic since these sources of information are not available. However, support for pathogenicity can be obtained in other ways, such as literature searches. Thus, family information is helpful but not necessary for determining variant pathogenicity in most cases.

We found that prediction is even more difficult in genes that are subject to more variation because of their increased size or tolerance for variation. Since functional

information was not available for most variants, 152/249 distinct variants (61.0%) were classified as VUS.

Some variants had been previously described in the dbSNP, EVS, and/or 1000 Genomes databases, and of 249 distinct variants, 126 (50.6%) were classified by at least one of the three databases used to assess variant classification (HGMD, ClinVar, and/or Emory). The available classifications in these cases were helpful but not always clear, mainly due to discrepancies among bioinformatic prediction databases or functional studies; low numbers of unrelated affected individuals with the same variant; misreporting of variant pathogenicity in the literature or classification databases; or occurrence of variants in large or highly mutable genes. These inconsistencies all point to the need for a single, centralized database and methodology for variant classification.

Our study has several limitations. First of all, many individuals in our cohort were ascertained for various Mendelian disorders. Because of this, two of the reportable findings could not be considered incidental, in that they may actually have contributed to the phenotype of the patients. In addition, our cohort was relatively small, and 73% of individuals self-identified with European descent. Moreover, we may have missed some variants due to low coverage in some regions of the 56 ACMG genes. Another limitation was the minor allele frequency cut-offs that we employed. For our early comparisons of variant classification between databases, we used a 1% MAF cut-off, which may be too high for some disorders and necessitate more time for manual review of variants. On the other hand, our later assessments of variant reportability used more stringent minor allele frequency cut-offs based on expected disease prevalences. This increased stringency may have resulted in exclusion of variants which were in fact pathogenic. In addition, the

method we used to find reportable variants fails to consider functional studies. Because of this, we may have excluded some variants which have been functionally characterized as pathogenic or included variants which show milder or less conclusive functional consequences.

An alternative sequencing method like whole-genome sequencing would increase detection of incidental findings as well as VUS, owing to the increased coverage of this method and the challenge of interpreting noncoding variants. Whole-genome sequencing will uncover structural variants and copy-number variants as well. However, the ACMG recommendations currently apply only to SNVs and indels, so this additional variation—some of which may be significant—would not be considered under the current guidelines (Green et al., 2013).

Based on this experience, we suggest that clinicians and researchers interested in returning incidental variants found by next-generation sequencing adopt a uniform, well-defined set of criteria for variant classification. Here we applied the ACMG recommendations and the classification criteria defined by Dorschner et al. (2013), but we suggest that the addition of bioinformatic prediction tools, genic intolerance scores, and functional studies to the criteria would make variant classification more complete. We also expect that research and clinical laboratories performing next-generation sequencing will soon be more engaged in submitting variants to manually curated classification databases, improving interpretation of variants and counseling of patients.

Acknowledgements

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Chapter 3 Figures

Figure 1. Classification of the 249 variants by the HGMD, ClinVar, and Emory databases.

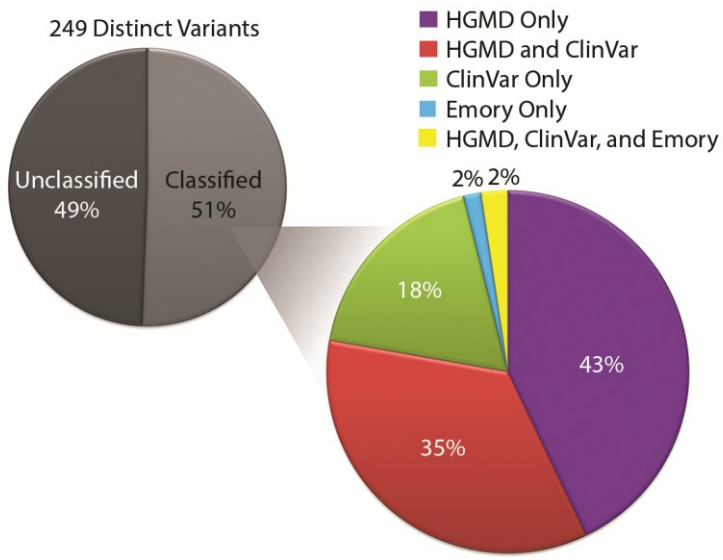
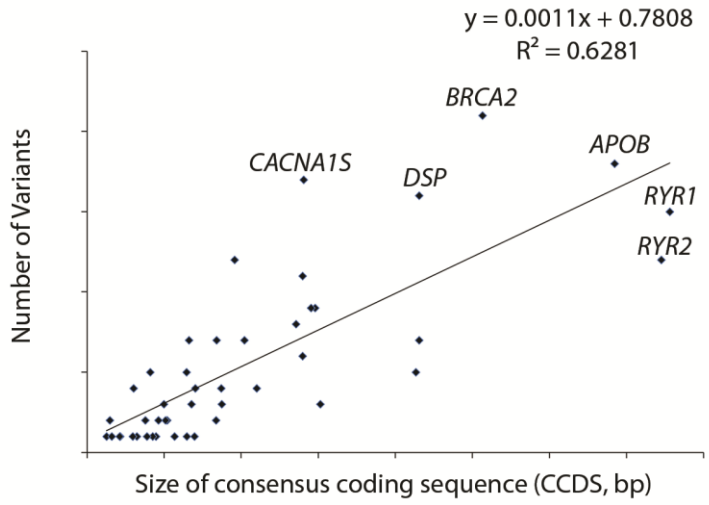


Figure 2. The number of variants per gene plotted against the size (kb) of the consensus coding sequence (CCDS) of the gene.



Chapter 3 Tables

Table 1. Classification of the 249 variants by mutation type.

Variant Type	Number of Variants
Missense SNV	231
Nonframeshifting indel	5
Nonsense	4
Exonic/Splicing	4
Frameshifting indel	3
Splicing	2
Total	**249**

Table 2. Reportable incidental variants identified in 232 individuals.

Gene	Chrom-osome	Genomic Change	cDNA & Protein Change	Criteria met	Classification	Reportable?	Incidental?	Patient ancestry
LMNA	1	156106743G>A	NM_005572.3: c.1412G>A, p.Arg471His	MAF < cutoff In 3 unrelated affected individuals (Astejada et al., 2007; Cowan, Li, Gonzalez-Quintana, Morales, & Hershberger, 2010; Parks et al., 2008)	Likely pathogenic VUS	Yes	No	European
MSH2	2	47656969C>T	NM_000251.2: c.1165C>T, p.Arg389*	MAF < cutoff Protein truncation in disease caused by haploinsufficiency (S. R. Payne & Kemp, 2005)	Pathogenic	Yes	Yes	Admixed African/ European
MUTYH	1	45798269T>C	NM_001048173.1: c.583A>G, p.Ile195Val	MAF < cutoff Segregation in one affected family (Morak, Massdorf, Sykora, Kerscher, & Holinski-Feder,	Likely pathogenic VUS	No	Yes	European

				2011) In one unrelated singleton (Sieber et al., 2003)				
MYBPC3	11	47356628G>C	NM_000256.3: c.2870C>G, p.Thr957Ser	MAF < cutoff In 3 unrelated affected individuals (Ehlermann et al., 2008; Olivotto et al., 2008; Rodríguez-García et al., 2010)	Likely pathogenic VUS	Yes	No	European
MYLK	3	123512573delG	NM_053025.3: c.116delC, p.Pro39Leufs*26	MAF < cutoff Protein truncation in disease caused by haploinsufficiency (Li Wang et al., 2010)	Pathogenic	Yes	Yes	Latino

Chapter 3 Supplement

Supplementary Table 1. Variation found in 56 ACMG genes in whole exome sequences of 232 individuals.

Whole exome sequences from 232 individuals representing 89 families were analyzed for rare (MAF<0.01), nonsynonymous and splice variants and indels in the 56 genes on the ACMG list (Green et al., 2013). We identified 249 distinct variants, some of which were seen in more than one individual. The number and density of variants (number of variants/kb coding sequence) varied by gene. Some genes had better sequencing coverage than others.

Gene name	Number of variants	Variant Density (variants/kb coding length)	Average coverage (read depth)	% of UCSC-defined gene (coding exons) with > 15 reads
BRCA2	21	2.05	119.19	100
APOB	18	1.31	145.84	99.3
CACNA1S	17	3.02	77.21	100
DSP	16	1.86	139.22	99.5
RYR1	15	0.99	70.55	95.9
MYBPC3	12	3.14	73.39	96
RYR2	12	0.81	97.18	100
BRCA1	11	1.97	128.26	98.6
MYH11	9	1.52	86.16	99.3
MYH7	9	1.55	89.83	99.3
TSC2	8	1.47	68.63	99.2
DSG2	7	2.09	117.28	98.6
FBN1	7	0.81	82.62	100
MSH6	7	1.71	140.95	100
PKP2	7	2.65	61.38	85.9
MYLK	6	1.07	86.89	99.4

APC	5	0.59	138.56	100
LDLR	5	1.94	97.36	100
MUTYH	5	3.05	100.4	100
COL3A1	4	0.91	60.42	97.6
KCNH2	4	1.15	57.09	93.4
MSH2	4	1.43	69.57	96.8
TMEM43	4	3.33	69.6	100
DSC2	3	1.11	88.23	99.2
LMNA	3	1.50	65.56	93.8
SCN5A	3	0.50	94.67	100
TSC1	3	0.86	79.03	100
KCNQ1	2	0.98	51.29	90.8
MEN1	2	1.08	88.65	97.5
MYL3	2	3.40	69.42	100
PCSK9	2	0.96	54.74	97.2
RET	2	0.60	73.72	98.8
TGFBR1	2	1.32	93.89	93.5
GLA	1	0.78	80.29	100
MLH1	1	0.44	83.88	99.9
NF2	1	0.56	69.81	99.8
PMS2	1	0.39	111.39	100
RB1	1	0.36	78.21	99.5
SDHAF2	1	2.00	89.02	100
SDHB	1	1.19	66.82	100
TGFBR2	1	0.59	89.8	100
TNNT2	1	1.17	74.71	99.9
TP53	1	0.85	75.77	100
VHL	1	1.56	83.57	100
WT1	1	0.64	56.33	100
ACTA2	0	N/A	76.42	100
ACTC1	0	N/A	90.69	99.1
MYL2	0	N/A	98.89	100
PRKAG2	0	N/A	70.88	100

PTEN	0	N/A	115.28	100
SDHC	0	N/A	45.33	92.4
SDHD	0	N/A	27.01	71.8
SMAD3	0	N/A	72.32	98.9
STK11	0	N/A	60.34	97.4
TNNI3	0	N/A	72.82	100
TPM1	0	N/A	77.35	100

Supplementary Table 2. Discordant classification of variants among databases.

Three databases (HGMD (Stenson et al., 2003), ClinVar (Landrum et al., 2014), and Emory (Bean et al., 2013)) were examined for classification of the 249 distinct variants identified in 232 individuals' whole exome sequences. Of 48 variants which were classified by more than one database, 22 variants were given discordant classifications between databases. We then compared these database classifications to our variant classifications derived from the Dorschner et al. method (Dorschner et al., 2013) and found that variant classifications given by the ClinVar and Emory databases but not HGMD were generally consistent with our variant classifications. These results show the lack of consensus for variant classification methodologies among currently available databases.
(Abbreviations: DM-Disease-causing (pathological) mutation, based on information from the literature; VUS-Variant of Uncertain Significance; N/A- variant was not listed in this database).

Gene (Transcript Accession Number)	**Chrom-osome**	**Genomic Change**	**cDNA & Protein Change**	**HGMD Classification**	**ClinVar Classification**	**Emory Classification**	**Classification from Dorschner et al. Method**	**Rationale**
BRCA1 (NM_007294.3)	17	41251803T>C	c.536A>G, p.Tyr179Cys	DM	No known pathogenicity	N/A	Likely benign VUS	Seen in combination with known pathogenic mutation (Judkins et

								al., 2005)
BRCA1 (NM_007294.3)	17	41245900T>G	c.1648A>C, p.Asn550His	DM	No known pathogenicity	N/A	Likely benign VUS	Seen in combination with known pathogenic mutation (Tavtigian et al., 2006)
BRCA1 (NM_007294.3)	17	41223048A>G	c.4883T>C, p.Met1628Thr	DM	Probably not pathogenic	N/A	Likely benign VUS	Seen in combination with known pathogenic mutation (Phelan et al., 2005)
BRCA2 (NM_000059.3)	13	32907129T>C	c.1514T>C, p.Ile505Thr	DM	Probably not pathogenic	N/A	VUS	MAF < prevalence of disorder In < 3 unrelated affected individuals (Cavallone et al., 2010; Edwards, Kote-Jarai, Hamoudi, & Eeles, 2001)
BRCA2 (NM_000059.3)	13	32914839A>G	c.6347A>G, p.His2116Arg	DM	Probably not pathogenic	N/A	VUS	MAF < prevalence of disorder In < 3 unrelated affected individuals (Landrum et al., 2014)
BRCA2 (NM_000059.3)	13	32937521G>A	c.8182G>A, p.Val2728Ile	DM	Probably not pathogenic	N/A	Likely benign VUS	MAF > prevalence of disorder
BRCA2 (NM_000059.3)	13	32953971C>T	c.9038C>T, p.Thr3013Ile	DM	No known pathogenicity	N/A	VUS	MAF < prevalence of disorder In < 3 unrelated affected individuals

								(Malone et al., 2000)
BRCA2 (NM_000059.3)	13	32954034A>G	c.9101A>G, p.Gln3034Arg	DM	Variant of unknown significance	N/A	VUS	MAF < prevalence of disorder In < 3 unrelated affected individuals (Landrum et al., 2014)
DSC2 (NM_004949.3)	18	28672114C>T	c.304G>A, p.Glu102Lys	DM	Variant of unknown significance	N/A	Likely benign VUS	MAF > prevalence of disorder
DSG2 (NM_001943.3)	18	29099850G>A	c.166G>A, p.Val56Met	DM	Probably not pathogenic	N/A	Likely benign VUS	MAF > prevalence of disorder
DSG2 (NM_001943.3)	18	29101156T>G	c.473T>G, p.Val158Gly	DM	Probably not pathogenic	N/A	Likely benign VUS	MAF > prevalence of disorder
DSG2 (NM_001943.3)	18	29126108T>G	c.2759T>G, p.Val920Gly	DM	Probably not pathogenic	N/A	Likely benign VUS	MAF > prevalence of disorder
DSP (NM_004415.2)	6	7575014C>T	c.2422C>T, p.Arg808Cys	DM	Variant of unknown significance	N/A	VUS	MAF < prevalence of disorder In < 3 unrelated affected individuals (Al-Jassar et al., 2011)
DSP	6	7580795C>G	c.4372C>G,	DM	Variant of	N/A	Likely benign	MAF > prevalence of disorder

(NM_004415.2)			p.Arg1458Gly		unknown significance		VUS	
MSH2 (NM_000251.2)	2	47641430C>T	c.815C>T, p.Ala272Val	DM	Variant of unknown significance	N/A	VUS	MAF < prevalence of disorder In < 3 unrelated affected individuals (Landrum et al., 2014; Samowitz et al., 2001)
MYBPC3 (NM_000256.3)	11	47357479C>T	c.2686G>A, p.Val896Met	DM	Probably not pathogenic	N/A	VUS	MAF < prevalence of disorder In < 3 unrelated affected individuals (Jääskeläinen et al., 2002; Moolman-Smook, De Lange, Bruwer, Brink, & Corfield, 1999)
MYBPC3 (NM_000256.3)	11	47356628G>C	c.2870C>G, p.Thr957Ser	DM	Variant of unknown significance	N/A	Likely pathogenic VUS	MAF < prevalence of disorder In 3 unrelated affected individuals (Ehlermann et al., 2008; Olivotto et al., 2008; Rodríguez-García et al., 2010)
MYH7 (NM_000257.3)	14	23902827C>T	c.115G>A, p.Val39Met	DM	Variant of unknown	N/A	VUS	MAF < prevalence of disorder In < 3 unrelated affected individuals

					significance			(Richard et al., 2003)
MUTYH (NM_00104-8173.1)	1	45797139G>A	c.1192C>T, p.Arg398Cys	DM	Variant of unknown significance	N/A	Likely benign VUS	MAF > prevalence of disorder
PKP2 (NM_004572.3)	12	33031395G>A	c.419C>T, p.Ser140Phe	DM	Probably not pathogenic	N/A	Likely benign VUS	MAF > prevalence of disorder
RET (NM_020975.4)	10	43595999C>A	c.166C>A, p.Leu56Met	DM	No known pathogenicity	VOUS	Likely benign VUS	MAF > prevalence of disorder
RYR2 (NM_001035.2)	1	237730059C>T	c.3407C>T, p.Ala1136Val	DM	No known pathogenicity	N/A	Likely benign VUS	MAF > prevalence of disorder

Chapter 4. Mutations in *PCYT1A*, encoding a key regulator of phosphatidylcholine metabolism, cause spondylometaphyseal dysplasia with cone-rod dystrophy

Chapter 4 is reprinted from the final accepted journal article:

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Julie Jurgens's Contributions: Targeted sequencing of *PCYT1A* in some families; Sanger validation and segregation analysis of variants; restriction digest validation of variant in one family; writing portions of manuscript and assistance with submission process; figure and table preparation; assistance with revision and reformatting.

Introduction

The spondylometaphyseal dysplasias (SMDs) are a group of about a dozen rare disorders characterized by short stature, irregular, flat vertebrae and metaphyseal abnormalities. Aside from spondylometaphyseal dysplasia Kozlowski type (MIM 184252) caused by mutations in *TRPV4* (MIM 605427) and spondyloenchondrodysplasia (MIM 607944) due to mutations in *ACP5* (MIM 171640), the genetic etiologies of SMDs are unknown (Warman et al., 2011). Two of these unexplained SMDs have ophthalmologic manifestations: SMD with cone-rod dystrophy (SMD-CRD [MIM 608940]) and axial SMD with retinal degeneration (MIM 602271).

Delineated clinically a decade ago, SMD-CRD is a presumed autosomal recessive disorder with postnatal growth deficiency leading to profound short stature; rhizomelia with bowing of the lower extremities; platyspondyly with anterior vertebral protrusions;

progressive metaphyseal irregularity and cupping with shortened tubular bones; and early onset, progressive visual impairment associated with a pigmentary maculopathy and electroretinographic evidence of cone-rod dysfunction (Kitoh et al., 2011; Sousa, Russell-Eggitt, Hall, Hall, & Hennekam, 2008; Turell, Morrison, & Traboulsi, 2010; Walters et al., 2004). In contrast to retinitis pigmentosa, the CRDs have early involvement of cone photoreceptors (Hamel, 2007).

Here, we report loss of function mutations in *PCYT1A* (MIM 123695) as the cause of SMD-CRD. *PCYT1A* encodes CTP:phosphocholine cytidylyltransferase (CCTα; Fagone & Jackowski, 2013; Kent, 2005), a key enzyme in the CDP-choline or Kennedy pathway for *de novo* phosphatidylcholine biosynthesis.

Subjects and Methods

We used whole exome and targeted sequencing of members of 6 unrelated families with 8 individuals with SMD-CRD (Figure 1). Three families were submitted to the Baylor-Hopkins Center for Mendelian Genomics (BHCMG) through the online submission portal PhenoDB (Hamosh et al., 2013) and, to confirm our observations in the first 3 families, we recruited 3 additional families for targeted candidate gene sequencing. The clinical features of these individuals are summarized in Table 1 and briefly reviewed here. Six of the subjects have been described in previous publications (Kitoh et al., 2011; Walters et al., 2004).

Subject 1 (BH2265_1; Family 1; Figure 2A) was reported when she was 20 y old (Walters et al., 2004). Now age 29 y, she has done well with continued linear growth to an adult height of 93.9 cm (-10.7 SD) and modest progression of limitation of range of motion. Visual impairment has not progressed since around age 10 y. Subject 2

(BH2283_1; Family 2, Figure 2E), originally described at age 11 y, is now 20 y old with a current adult height of 139 cm (-5.3 SD) and some progression of joint stiffening. His visual function declined during his second decade and he now requires low vision aids. Subject 3 (BH2233_1; Family3) is a previously unreported 61-year-old female who was first seen at age 51. Although her skeletal phenotype is similar to that of the others described here, she had a late-onset retinal phenotype (Figure 2B). Radiographs from childhood were reported to show platyspondyly and metaphyseal changes; adult radiographs show hypoplasia of the posterior vertebral bodies but no anterior vertebral protrusions. Adult height (measured at age 54 y) is 108.3 cm (-8.4 SD). Visual symptoms were not apparent until middle age. An ERG at age 43 y (performed because she had a brother with visual impairment) was said to be normal. By age 51 y, however, her ERG showed evidence of cone-rod dysfunction and her current vision is limited (Table 1). At age 54 y, her examination showed short stature, rhizomelic limb shortening, brachydactyly, stiffness of large joints and internal tibial torsion. Her family history includes an affected brother, who died at age 45 y and was known to have short stature and a confirmed CRD. Her parents may be distantly related. Subjects 4-6 were reported by Walters et al.[2] as their cases 3-5, and have not been formally assessed since that time but provided updated information regarding visual function (Table 1, Figure 2C and D). Subject 7 (Family 5) was previously described (Kitoh et al., 2011). Subject 8 (Family 6) is a 23 m old previously unreported Korean male referred for evaluation of growth failure and disproportionate shortening of the limbs. He has an increased antero-posterior thoracic diameter, rhizomelic shortening of his extremities and bilateral bowing of the legs, and mildly limited elbow extension, knee extension, and hip abduction. No visual or

hearing impairment was noted at his first examination. Linear growth was impaired (by North American standards): at 23 m he was 68.4 cm (-5.2 SD), and at 48 m 71.2 cm (-7.4 SD). From age 2 y he had frequent pneumonias with episodes of desaturation and O_2 dependency thought to be due to chest wall deformity. He had a waddling gait due to coxa vara deformity. Radiographs at 23 m showed short, bowed long bones with flared, cupped, and spurred metaphyses, and the adjacent epiphyses were large and rounded. In the hands and feet, the metaphyses of the short tubular bones had mild cupping, widening, and flaring, and the diaphyses were short. The vertebral bodies were ovoid, mildly flattened, with anterior projections. These radiographic abnormalities were more severe at age 45 m. Mild scoliosis developed. Although no visual impairment was noted at age 23 m by age 45 m fundus examination showed hypopigmented macular atrophy in both eyes with markedly decreased photopic and moderately decreased scotopic ERGs.

For our molecular studies, we isolated genomic DNA from fresh whole blood using the Gentra Puregene Kit (Qiagen Sciences, Germantown, MD). Subjects 1, 2 and 3 were genotyped on Illumina's ExomeChip1.1 GWAS array. This allows us to estimate inbreeding coefficient based on the observed and expected homozygous genotypes at genome wide level among 199 unrelated Caucasian samples (defined by PCA) after LD pruning (PLINK). The inbreeding coefficient was 0.1535, -0.007 and 0.035 for subjects 1, 2 and 3 respectively, suggesting subject 1 (BH2265_1) is the product of an unrecognized consanguineous union. Although subject 3 is also homozygous for p.A99V mutation, B allele frequency plot showed multiple loss-of-heterozygosity segments across genome for subject 1 but not subject 3. This result suggests that homozygosity for p.A99V in subject 1 is a result of consanguinity while in subject 3 it is the result of recurrent mutation (see

below).

For WES, we captured the CCDS exonic regions and flanking intronic regions totaling ~51Mb using the Agilent SureSelect Human All Exon V4 51Mb Kit and performed paired end 100 bp reads on subjects 1-3 using the Illumina HiSeq2000 platform. We aligned each read to the reference genome (NCBI human genome assembly build 37; Ensembl core database release 50_361; Hubbard et al., 2009) using the Burrows-Wheeler Alignment (BWA) tool (H. Li & Durbin, 2009) and identified single nucleotide variants (SNVs) and small insertion-deletions (indels) using SAMtools (H. Li et al., 2009). We also performed local realignment and base call quality recalibration using GATK (DePristo et al., 2011; McKenna et al., 2010). We identified potentially causal variants using standard filtering criteria: SNV and indel minimal depth of 8X, root mean square mapping quality of 25, strand bias p-value below 10^{-4}, end distance bias below 10^{-4}, and filtering out SNVs within 3bp of an indel and indels within 10bp of each other; followed by the use of the Analysis Tool of PhenoDB (Hamosh et al., 2013) to design the prioritization strategy (Sobreira, personal communication). We prioritized rare functional variants (missense, nonsense, splice site variants and indels) that were homozygous or compound heterozygous in each of the 3 subjects and excluded variants with a MAF > 0.01 in dbSNP 126, 129, and 131 or in the Exome Variant Server (release ESP6500SI-V2) or 1000 Genomes Project (1000 Genomes Project Consortium et al., 2012). We also excluded all variants found in our in-house controls (CIDRVar 51Mb). We generated a homozygous and a compound heterozygous variant list for each subject and merged them to identify genes that were mutated in both alleles in all three subjects. We designed PCR primers to amplify exons and flanking intronic splice sites followed by

direct Sanger sequencing to validate candidate causative variants, determine their segregation within families, and to sequence *PCYT1A* in subjects 4-8. All the variants described here were based on the Refgene transcript NM_005017.2 and NCBI human genome assembly build 37 (Table 1).

Results

Analysis of WES data in subjects 1-3 identified 2 genes containing candidate causal mutations in all 3 subjects: *TTN* (MIM 188840) and *PCYT1A*. *TTN* is a large gene (313 exons) expressed primarily in skeletal and cardiac muscle (Y. Zhang et al., 2007). *TTN* variants are found frequently in controls (Lopes et al., 2013) and have been implicated as causative in various cardiac and skeletal myopathies (Carmignac et al., 2007; Gerull et al., 2002; Hackman et al., 2002; D. S. Herman et al., 2012; Itoh-Satoh et al., 2002; Lange et al., 2005; Lopes et al., 2013; Satoh et al., 1999), but not in individuals with retinal or skeletal dysplasia phenotypes. For these reasons, we removed *TTN* from consideration, leaving *PCYT1A* as the only gene with rare variants in both alleles in all 3 subjects. Subjects 1 and 3 are homozygous for the *PCYT1A* missense variant p.Ala99Val (c.296C>T) in exon 5 and subject 2 is a compound heterozygote for p.Ala99Val and p.Pro150Ala (c.448C>G) in exon 6 of *PCYT1A*. These variants are not present in the >6,000 individuals in the Exome Variant Server nor in the 1092 individuals whose sequence is currently available from the 1000 Genomes. Direct Sanger sequencing of PCR amplified products confirmed appropriate Mendelian segregation of these variants in available family members (Figure 1). Based on these results, we used PCR and bidirectional Sanger sequencing to interrogate all *PCYT1A* exons and flanking intronic sequence in subjects 4-8 (3 probands and 2 affected sibs). All were compound

heterozygotes for rare *PCYT1A* variants.

Thus, in total, we identified 8 rare *PCYT1A* variants (1 nonsense, 1 frame shifting indel, and 6 missense variants) present either in the homozygous or compound heterozygous state in 8 individuals with SMD-CRD in 6 families from around the world (Table 1). The missense mutations all change highly conserved residues, including one, Ala99, that is altered by two different variants (p.Ala99Val, p.Ala99Thr) and all are predicted to be damaging by SIFT (Kumar et al., 2009) and either probably damaging or possibly damaging by PolyPhen-2 (Table 1; Adzhubei et al., 2010). The c.296C>T variant producing the p.Ala99Val change occurs at a CpG dinucleotide on the reverse strand, while, the mutation in the same codon producing p.Ala99Thr (c.295G>A) in family 4 does not involve a CpG dinucleotide. To determine if the three unrelated subjects with the p.Ala99Val change have a shared or recurrent mutation, we utilized the SNP genotyping data to analyze IBD sharing and runs of homozygosity to show that, as predicted by the different geographical origins of the 3 families, the p.Ala99Val variants in families 1-3 are on different haplotypes indicating that recurrent CpG mutations are responsible.

Discussion

Located at 3q29, *PCYT1A* (Figure 3) contains 10 exon, is ubiquitously expressed and encodes CCTα, an amphitropic enzyme that catalyzes the synthesis of CDP-choline from phosphocholine and CTP. The CCTα reaction is the rate-limiting step in the major pathway for phosphatidylcholine synthesis (Figure 4; Fagone & Jackowski, 2013; Kent, 2005). In mammals, phosphatidylcholine can also be synthesized from phosphatidylethanolamine in a reaction catalyzed by phosphatidylethanolamine N-

methyltransferase (PEMT) but the expression of the enzyme is limited to liver (Figure 4; Fagone & Jackowski, 2013; Glunde, Bhujwalla, & Ronen, 2011; Kent, 2005). Phosphatidylcholine is the predominant phospholipid in eukaryotic membranes (Fagone & Jackowski, 2013; Kent, 2005). Consistent with the importance of CCTα in phosphatidylcholine synthesis, a mouse knockout of *Pcyt1a* is an early embryonic lethal in the homozygous state (L. Wang, Magdaleno, Tabas, & Jackowski, 2005). An X-linked *PCYT1A* paralog*, PCYT1B* (MIM 604926)*,* encodes three isoforms CCTβ1, CCTβ2 and CCTβ3 with activities similar to CCTα but whose expression is limited to the central nervous system (Carter, Demizieux, Campenot, Vance, & Vance, 2008; Fagone & Jackowski, 2013).

The 367 residue CCTα has 4 domains: a 73 residue N-terminal domain containing the nuclear localizing signal, a 163 residue catalytic domain, a 64 residue membrane-binding M domain, and a C-terminal 67 residue unordered domain with multiple residues that undergo phosphorylation (Figure 3; Ding et al., 2012; J. Lee, Johnson, Ding, Paetzel, & Cornell, 2009). CCTα is found as an inactive homodimer in the nucleoplasm where, in response to lipid signaling, it binds to the nuclear membrane through interactions mediated by the amphipathic M domain and becomes catalytically active (Fagone & Jackowski, 2013; J. Lee et al., 2009). The sequence and domain structure of CCTα is highly conserved across phylogeny and the crystal structure of the catalytic domain of rat CCTα, which has 100% sequence identity with human CCTα, has been solved (J. Lee et al., 2009).

The *PCYT1A* missense alleles identified in the subjects with SMD-CRD all involve residues conserved across vertebrate and most of invertebrate and prokaryotic

phylogeny. All mutations change residues in the catalytic domain of CCTα: Ala99 (altered in subjects 1, 2, 3, 4, 5 and 6) is adjacent to Gln98 which is directly involved in binding CDP; Pro150 (altered in proband 2) is adjacent to Trp151 which is also directly involved in binding choline; Phe191 (altered in proband 8) is located 3 residues N-terminal to Thr194 which plays a role in coordinating CDP. The p.Glu129Lys and p.Arg223Ser substitutions alter residues that are linearly more distant from those that interact directly with substrates (J. Lee et al., 2009). The nonsense mutation, c.847C>T, occurs in the penultimate exon 9, 50 bp from the 3' end of the exon and possibly results in nonsense mediated RNA decay of the mutant transcript. The 1 bp indel allele in subject 7 (c.990delC) changes the frame and creates a longer transcript with 102 extra amino acids (p.Ser331Profs*140). We conclude that these variants, on the basis of the their extremely low frequency, the high conservation of the involved residues and predicted deleterious consequences for enzymatic activity, their occurrence in homozygosity or compound heterozygosity in multiple unrelated affected individuals in six families and their segregation fitting an autosomal recessive model, are *PCYT1A* loss of function alleles responsible for the SMD-CRD phenotype.

Like the deficiency of choline kinase B caused by mutations in *CHKB* (MIM 612395) responsible for muscular dystrophy, congenital megaconial type (MIM 602541; Mitsuhashi et al., 2011), SMD-CRD seems to be another inborn error in the phosphatidylcholine synthetic pathway. The central importance of this lipid in membrane biology suggests that complete loss of function of this pathway would result in embryonic lethality, as observed in mice homozygous for *Pcyt1a* null alleles (L. Wang et al., 2005). Thus, it is puzzling that individuals with SMD-CRD are healthy aside from

their skeletal and retinal involvement. One possible explanation is that despite the predicted severe functional consequences of the mutations we describe, there remains some residual CCTα function. This may be augmented by activity of the CCTβ isozymes and by synthesis of phosphatidylcholine from phosphatidylethanolamine by the PEMT catalyzed reaction in the liver (Fagone & Jackowski, 2013). The activities of the CCTβ isozymes and PEMT were increased in cells derived from conditional *Pcyt1a* knockout mice (Jacobs, Devlin, Tabas, & Vance, 2004; D. Zhang et al., 2000). These possibilities should be explored as they may suggest therapeutic strategies that could be effective, especially in younger SMD-CRD individuals.

Our results suggest a connection between bone and retinal lipid metabolism that would explain the sensitivity of these two tissues to deficiency of CCTα and impaired phosphatidylcholine synthesis. Photoreceptors have an especially high demand on membrane biosynthesis due to the daily shedding of outer segment discs (LaVail, 1976). Thus, it is not surprising that these cells might be especially susceptible to defects in the biosynthetic pathway for the most abundant membrane phospholipid. Indeed, photoreceptor degeneration in the *rd11* mouse has been shown to be due to loss of function mutations in the gene encoding lysophosphatidylcholine acyltransferase 1 (*Lpcat1*), a phospholipid remodeling enzyme (Bridges et al., 2010; Friedman et al., 2010). Interestingly, bone formation is abnormal in certain Mendelian disorders caused by mutations in genes encoding proteins involved in fatty acid metabolism, including rhizomelic chondrodysplasia punctata (MIM 215100; Braverman et al., 2002) and the Conradi-Hunermann form of chondrodysplasia punctata (MIM 302960; G. E. Herman et al., 2002). These observations suggest key functions for membrane lipids in bone

formation and homeostasis.

Among our families there was no obvious genotype-phenotype correlation apparent from evaluation of the 6 affected individuals in 4 families with mutations altering codon Ala99. Subjects 1 and 3, both homozygous for c.296C>T, p.Ala99Val, have strikingly dissimilar age at onset of visual phenotype, of clinical characteristics and of radiologic features. This broad range of phenotypic severity in individuals with the same mutations at the disease gene locus suggests that variation in other components of the phosphatidylcholine pathways and/or environmental variables may have a strong influence on phenotypic manifestations and severity. Understanding these variables may lead to improved management of individuals with SMD-CRD. Interestingly, one of the individuals described here, subject 8, has had frequent pneumonias and has required supplemental oxygen from age 2 y. We speculate that this might relate to the high demand for phosphatidylcholine in surfactant biosynthesis by the alveolar type II cells (Agassandian & Mallampalli, 2013; Goss, Hunt, & Postle, 2013). These cells are known to express *PCYT1A* at high levels and phosphatidylcholine is the major phospholipid component of surfactant (Agassandian & Mallampalli, 2013; Goss et al., 2013; Ridsdale, Tseu, Wang, & Post, 2001). A conditional, epithelial cell-specific *Pcyt1a* knockout mouse had severe respiratory failure at birth and reduced levels of surfactant (Tian, Zhou, Rehg, & Jackowski, 2007). Although only one of the individuals described here has had this problem, the potential biological connections suggest that additional studies of pulmonary function and surfactant status in proband 8 and possibly other cases of SMD-CRD is warranted.

To our knowledge, SMD-CRD is the only described disorder in which CRD is

associated with a skeletal dysplasia. By contrast, there are several examples of RP associated with bony abnormalities including some for which the responsible gene is yet to be identified (Ehara, Kim, Maisawa, Takasago, & Nishimura, 1997; Isidor, Le Merrer, Ramos, Baron, & David, 2009). CRD and RP may be part of the same phenotypic spectrum of retinal degeneration. Mutations in certain genes have been reported to cause either phenotype (Cremers et al., 1998; Yang et al., 2008). For this reason, we suggest that *PCYT1A* should be assessed in any individual with CRD or RP associated with any form of spondylometaphyseal dysplasia or spondyloepiphyseal dysplasia, including in particular, individuals with the axial SMD with retinal degeneration phenotype (MIM 602271).

Acknowledgements

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Web Resources

The URLs for data presented herein are as follows:

Baylor-Hopkins Center for Mendelian Genomics, http://mendeliangenomics.org.

Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA, http://evs.gs.washington.edu/EVS/.

1000 Genomes, http://www.1000genomes.org.

Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/.

Picard software, http://picard.sourceforge.net.

PLINK, http://pngu.mgh.harvard.edu/~purcell/plink/ibdibs.shtml.

PolyPhen-2, http://www.genetics.bwh.harvard.edu/pph2/

SIFT, http://sift.bii.a-star.edu.sg/

Chapter 4 Figures

Figure 1. Pedigrees of families 1-6 showing the segregation of *PCYT1A* mutant alleles. Alleles with the wild type genotype indicated by "+". Samples were not available for individuals lacking a genotype designation.

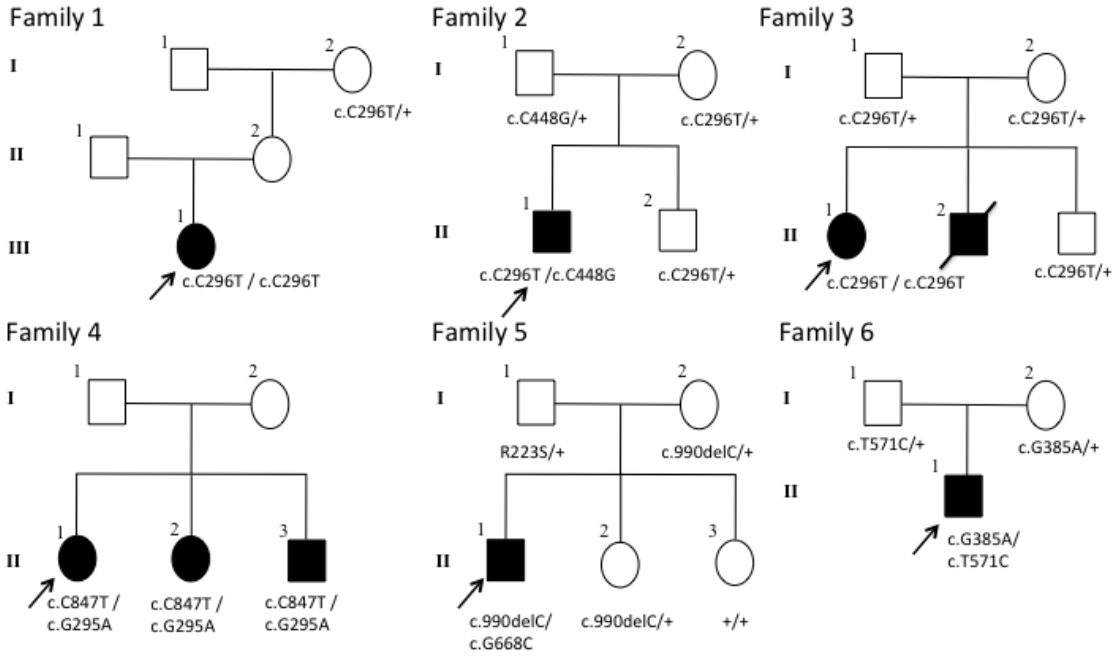


Figure 2. Clinical and radiographic features of SMD-CRD. A – General physical phenotype (Subject 1 at age 16 y 5 m); B – Fundus photograph showing pigmentary maculopathy (Subject 3 at age 61 y); C – Spine and pelvis radiograph demonstrating platyspondyly, characteristic pelvic configuration and proximal femoral metaphyseal changes (Subject 6 at 3 y 8 m of age); D – Pelvis and femurs showing the marked metaphyseal changes that are typical (Subject 4 at age 11 y 1 m); E – Knee radiographs, similarly showing the marked metaphyseal changes that are typical in this diagnosis (Subject 2 at 13 y).

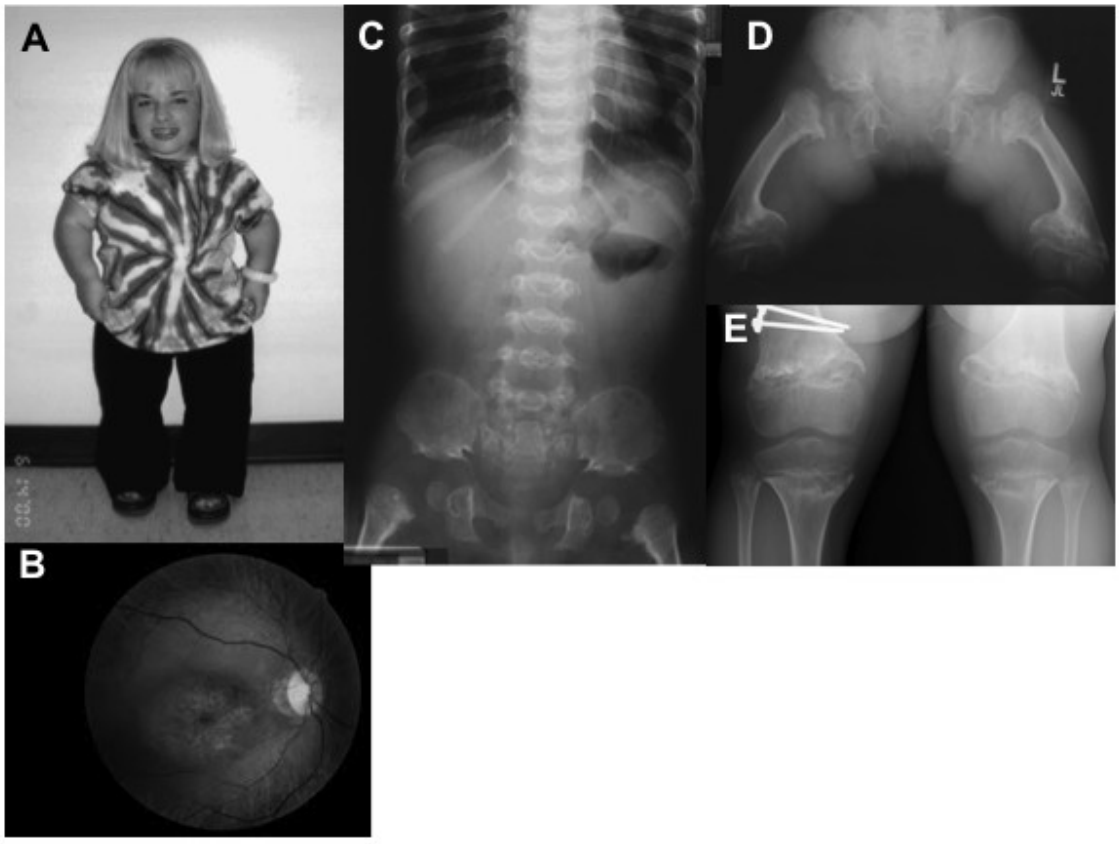


Figure 3. *PCYT1A* structure and domain organization of CCTα. *PCYT1A* is located at 3q29 and includes 10 exons (gray rectangles) as shown on the top line of the diagram. The thin rectangles encode the 5' and 3' UTR sequences. The right angle arrow denotes the start of the open reading frame and the asterisk indicates the location of the stop codon. The genomic coordinates on chromosome 3 are shown above. Below in color is a diagram of CCTα showing the domain structure with the diagonal dashed lines indicating the exons that contribute to each domain. The N-terminal domain (N) is in light blue with a dark blue rectangle indicating the position of the nuclear localization signal. The catalytic domain (C) is in pink. The amphitrophic membrane-binding domain (M) is in green and the C-terminal phosphorylated domain (P) is in purple. The numbers below indicate the residues at the boundaries of the domains. The mutations and their locations are shown below.

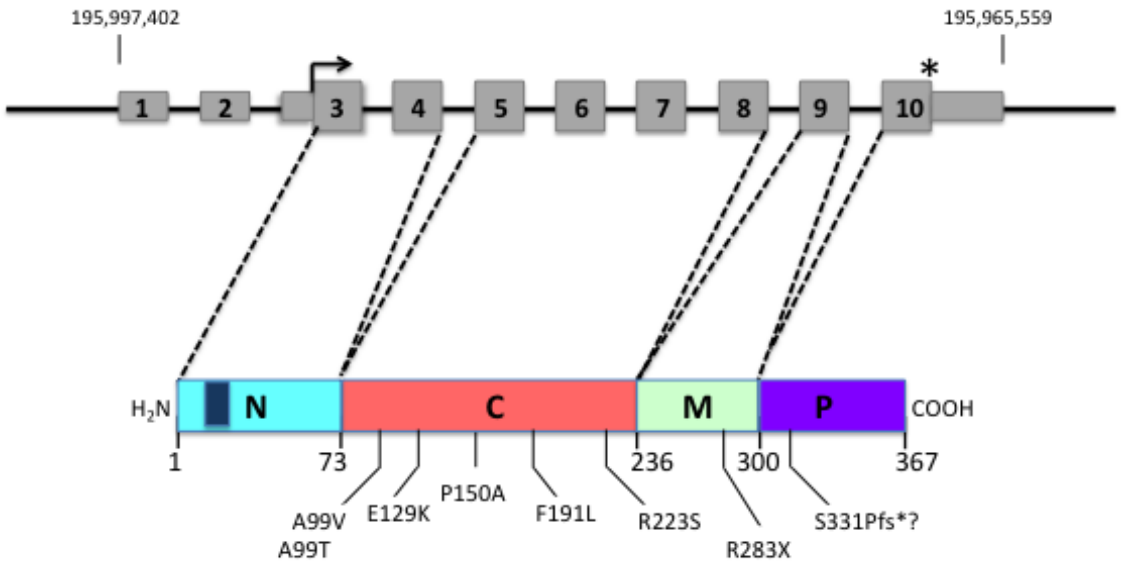
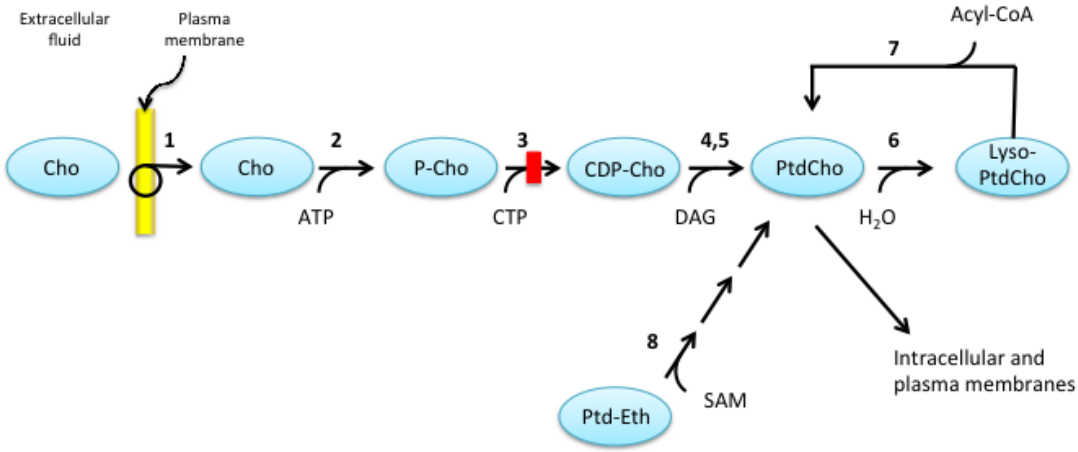


Figure 4. Pathways of phosphatidylcholine biosynthesis. In this figure, enzymes are indicated by numbers as defined below. Choline (Cho) is transported from the extracellular fluid across the plasma membrane by Na^+-dependent, ATP-requiring choline transporter-like proteins (1). Free intracellular choline is phosphorylated by choline kinase (2) to produce phosphocholine (P-Cho). The latter is converted to cytidine-diphosphate choline (CDP-Cho) in a reaction catalyzed by phosphocholine cytidylyltransferase (CCTα, 3). CDP-choline is esterified with diacylglycerol (DAG) to phosphatidylcholine (PtdCho) in reactions catalyzed either by cholinephosphotransferase (4) or choline/ethanolaminephosphotransferase (5). PtdCho is converted to lyso-phosphatidylcholine (Lyso-PtdCho) by phospholipase A (6) which, in turn, is converted back to PtdCho, in a reaction catalyzed by acylglycerophosphate acyltransferase (7). In mammals, a second pathway of PtdCho synthesis is limited to liver, where PtdCho is synthesized from phosphatidylethanolamine (Ptd-Eth) in a series of methylation reactions catalyzed by phosphatidylethanolamine *N*-methyltransferase (8) with S-adenosylmethionine (SAM) as the methyl donor. The red rectangle indicates the position of the block in PtdCho synthesis caused by deficiency of CCT.



Chapter 4 Tables

Table 1. Demographic, Clinical and Molecular Findings

	Family 1	**Family 2**	**Family 3**	**Family 4**			**Family 5**	**Family 6**
	Subject 1 (BH2265_1)	**Subject 2 (BH2283_1)**	**Subject 3 (BH2233_1)**	**Subject 4**	**Subject 5**	**Subject 6**	**Subject 7**	**Subject 8**
Previously reported	Walters et al., 2004 Case 1	Walters et al., 2004 Case 2	No	Walters et al., 2004 Case 3	Walters et al., 2004 Case 4	Walters et al., 2004 Case 5	Kitoh et al., 2011	No
Family origin	North Europe	North Europe	Greece	North Europe	North Europe	North Europe	Japan	Korea
Age of initial recognition	7 m	13 m	51 y	36 m	27 m	24 m	6 m	23 m

Best corrected visual acuity	20/100	20/180	8/80	5/250	20/250	10/250	NA	NA
Pigmentary maculopathy	+	+	+	+	+	+	+	+
Cone-rod dystrophy (age of diagnosis)	+ (13½ y)	+ (17 m)	+ (51y)	+ (36 m)	+ (27 m)	ND	+ (11 y)	+
Height SD (most recent measurement available)	-10.7 SD	-5.3 SD	-8 .4 SD	NA	-6.1 SD	NA	-7.9 SD	-7.4 SD

Bowing of long bones	+	+	+	+	+	+	+	+
Platyspondyly	+	+	+	+	+	+	+	+
Metaphyseal irregularity and cupping	+	+	+	+	+	+	+	+
PCTY1A genotype	c.296C>T/ c.296C>T	c.296C>T/ c.448C>G	c.296C>T/ c.296C>T	c.847C>T/ c.295G>A	c.847C>T/ c.295G>A	c.847C>T/ c.295G>A	c.990delC/ c.669G>C	c.385G>A/ c.571T>C
PCTY1A protein change	p.Ala99Val/ p.Ala99Val	p.Ala99Val/ p.Pro150Ala	p.Ala99Val/ p.Ala99Val	p.Arg283X/ p.Ala99Thr	p.Arg283X/ p.Ala99Thr	p.Arg283X/ p.Ala99Thr	p.Ser331Profs*140/ p.Arg223Ser	p.Glu129Lys/ p.Phe191Leu

SIFT / PolyPhen	0.04/1	0.04/1 0/1	0.04/1	0/1	0/1	0/1	0/0.791	0.02/1 0/1

Chapter 5:

Loss of function in *PCYT1A* has broad and cell-type specific consequences on lipid metabolism, chondrocyte differentiation, and lipid droplet formation

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Julie Jurgens's Contributions: Conceptualization and execution of all experimental procedures, data analysis and interpretation for all figures and tables, preparation of manuscript and all figures and tables

Introduction

Spondylometaphyseal dysplasia with cone-rod dystrophy (SMD-CRD) is a rare, autosomal recessive disorder of the skeleton and the retina. Clinical characteristics include progressive, early-onset photoreceptor degeneration—particularly in the macula—as well as short stature, bowing of the long bones, metaphyseal flaring, rhizomelic shortening, platyspondyly, and scoliosis. Using whole exome sequencing and Sanger sequencing, Hoover-Fong et al. (2014) and Yamamoto et al. (2014) identified homozygous or compound heterozygous variants in *PCYT1A* as the cause of SMD-CRD (Hoover-Fong et al., 2014; Yamamoto et al., 2014). These variants ranged from missense (RefSeq NM_005017.2: c.296C>T, p.Ala99Val; c.295G>A, p.Ala99Thr; c.385G>A, p.Glu129Lys; c.448C>G, p.Pro150Ala; c.571T>C, p.Phe191Leu; and c.669G>C, p.Arg223Ser) to nonsense variants (c.847C>T, p.Arg283Ter) or frameshifting indels (c.990delC, p.Ser331Profs*?; c.968dupG, p.Ser323Argfs*38; c.996delC, p.Ser333Leufs*?). Since the publication of these initial reports, our group has identified

two additional missense alleles in *PCYT1A* in patients with SMD-CRD (c.341G>C, p.Ser114Thr and c.718T>C, p.Tyr240His; Jurgens et al., personal communication).

Interestingly, compound heterozygous *PCYT1A* variants were also detected in two unrelated probands with congenital lipodystrophy and fatty liver disease (F. Payne et al., 2014). These patients had no ascertainable retinal or skeletal findings, suggesting a distinct pathophysiological mechanism. Both patients shared an in-frame deletion variant (c.838_840delCTC, p.Glu280del) in trans to a second variant in the other allele (c.424G>A, p.Val142Met or c.996delC, p.Ser333Leufs*164, respectively). Testa et al. recently reported a third class of patients with biallelic *PCYT1A* variants and retinal degeneration, but no accompanying skeletal dysplasia or lipodystrophy (Testa et al., 2017). Both probands shared the same *PCYT1A* missense variant (c.277G>A, p.Ala93Thr) in trans to an inactivating variant in the second allele (c.897+1G>A or c.847C>T, p.Arg283Ter, respectively).

PCYT1A encodes CTP:phosphocholine cytidylyltransferase α (CCTα), which catalyzes the rate-limiting step in *de novo* synthesis of phosphatidylcholine (PC) by the Kennedy pathway. *PCYT1A* is expressed ubiquitously in mice and in humans, and PC is the predominant membrane phospholipid in mammalian cells (R. B. Cornell & Ridgway, 2015; Karim, Jackson, & Jackowski, 2003; Keenan & Morré, 1970; L. Wang, Magdaleno, Tabas, & Jackowski, 2005; GTEx project release v7). Complete loss of *Pcyt1a* expression has devastating consequences: *Pcyt1a* $^{-/-}$ mice experience embryonic lethality (L. Wang et al., 2005), and MT-58 CHO cell lines with a 95% loss-of-function point mutation in *Pcyt1a* undergo apoptosis (Cui et al., 1996; Sweitzer & Kent, 1994). Based

on this evidence, PC production by the Kennedy pathway appears to be essential for life, at least in these model systems.

Though most PC is produced by the CCTα-dependent Kennedy pathway in mammalian cells, there are other compensatory pathways for PC production in mammals (R. B. Cornell & Ridgway, 2015; Jackowski et al., 2004; Fig. 2). *PCYT1B*, a paralog of *PCYT1A*, encodes the enzyme CCTβ, which catalyzes the same Kennedy pathway reaction as CCTα but has limited expression in the brains and reproductive tissues of mice and humans (Jackowski et al., 2004; Karim et al., 2003; GTEx project v7). Independently of the Kennedy pathway, PC can be formed by sequential methylation of another phospholipid, phosphatidylethanolamine (PE), by the PEMT pathway, which functions primarily in liver and adipocytes (Hörl et al., 2011; Kennedy & Weiss, 1956). Finally, in a series of reactions known as the Lands cycle, PC can either be converted into lysophosphatidylcholine (LPC) through fatty acid removal catalyzed by phospholipase A_2 (PLA_2), or LPC can be reacylated to form PC by lysophosphatidylcholine acyltransferases (LPCATs) (Lands, 1957; Vance & Vance, 2004). Interestingly, rd11 mice with *Lpcat1* deletions have a retinal degeneration phenotype, bolstering support for involvement of this arm of the phospholipid metabolism pathway in retinal degeneration (Friedman et al., 2010).

CCTα has a variety of functional domains which regulate its activity, conformation, and localization (R. B. Cornell & Ridgway, 2015). First, CCTα possesses a catalytic domain which is responsible for catalyzing PC production by the Kennedy pathway. In addition, an N-terminal nuclear localization signal localizes CCTα to the nucleus in most cells, where it exists diffusely throughout the nucleoplasm under basal conditions. Upon

activation, CCTα undergoes a series of conformational changes mediated by a membrane-binding domain (M domain) and a C-terminal phosphorylation domain in order to bind the nuclear lamina. Membrane binding elicits catalytic activation of the enzyme. It is worth noting that most SMD-CRD variants are missense variants within the catalytic domain of the enzyme (Fig. 1), although two variants fall in the M domain (p.Tyr240His and p.Arg283Ter) and three frameshifting indels localize to the C-terminal phosphorylation domain (p.Ser331Profs*?, p.Ser323Argfs*38, and p.Ser333Leufs*?).

CCTα has a number of interesting features from a cell biology standpoint, including its relationship with cellular organelles called lipid droplets (LDs). LDs form in response to loading with neutral lipids (e.g. oleate) in order to promote lipid storage and to protect against cytotoxicity (Walther, Chung, & Farese, 2017). Phospholipids such as PC are required for the formation of LD membranes, which encapsulate neutral lipids and prevent their coalescence (Krahmer et al., 2011). Some neutral lipids activate CCTα and stimulate PC production; for instance, oleate facilitates the dephosphorylation and membrane translocation of CCTα, leading to its activation (Aitchison, Arsenault, & Ridgway, 2015; Cornell & Vance, 1987; Gehrig, Cornell, & Ridgway, 2008; Lagace & Ridgway, 2005; Pelech, Pritchard, Brindley, & Vance, 1983; Y. Wang, Macdonald, & Kent, 1993). Accordingly, Drosophila, mouse, and rat cells deficient in CCTα orthologs accumulate fewer and larger lipid droplets as compared to their wild-type counterparts in response to oleate loading (Aitchison et al., 2015; Guo et al., 2008; Krahmer et al., 2011).

Here we describe the generation and characterization of three distinct cellular model systems—cultured dermal fibroblasts, HEK293 cells, and ATDC5 cells—to interrogate diverse functional consequences of SMD-CRD variants in *PCYT1A*. Using cultured skin

fibroblasts obtained from SMD-CRD patients, we sought to understand the consequences of SMD-CRD variants on cellular phenotypes in an endogenous context. Next, we generated *PCYT1A*-null HEK293 cell lines in order to assess the impact of variants on previously described cell biology phenotypes such as lipid droplet formation. Finally, we created a *PCYT1A*-null ATDC5 (pre-chondrocyte) cell lines, which are putatively more relevant to SMD-CRD pathophysiology and allowed us to measure the effects of *PCYT1A* variants on chondrocyte proliferation and differentiation. Using a combination of biochemical, cellular, and lipidomic approaches, we determined that SMD-CRD variants or complete loss of *PCYT1A* expression results in highly variable and cell-type specific phenotypic consequences.

Materials and Methods

Patient consent

Our study was approved by the Johns Hopkins Medicine Institutional Review Board and by the IRBs of other participating institutions. We obtained informed consent from all responsible individuals who participated in this study.

Cell Culture

Primary adherent fibroblast cells were derived from skin biopsies of SMD-CRD patients or wild-type controls. ATDC5 cells were a gift from Dr. Jill Fahrner of Johns Hopkins University (ECACC, Cat#99072806). HEK293T cells were a gift from Dr. Jeremy Nathans of Johns Hopkins University. CHO-K1 cells were a gift from Dr. James Phang of the NIH (ATCC, Cat#CCL61). All cell lines were cultured at 37° C under 5% CO_2 and passaged using 1 x trypsin-EDTA (Gibco, 15400-054). HEK293, CHO, and fibroblast cell lines were cultured in 1 x MEM (Gibco, 11430-030) supplemented with

non-essential amino acids (Gibco, 11140-050), 1% L-glutamine (Gibco, 25030-149), and 10% fetal bovine serum (Gemini Biosciences, 100-106). For experiments using delipidated serum, culture medium was the same, except 10% delipidized fetal bovine serum (Gemini Biosciences, Cat#900-123) was substituted for regular fetal bovine serum.

Prior to chondrocyte differentiation, ATDC5 cells were maintained in DMEM/F12 50/50 (Corning Cellgro, 15-090-CV) with 1% L-glutamine (Gibco, 25030-149), and 5% fetal bovine serum (Gemini Biosciences, 100-106), and 1% penicillin-streptomycin (Gibco, 15140-122). Chondrocyte differentiation was induced by adding 1 x insulin/transferin/selenium (Corning Cellgro, 25-800-CR) to the culture medium for 21 days.

DNA extraction and Sanger sequencing

DNA was extracted from blood or cell lines using the Puregene Blood Core Kit B (Qiagen, Cat#158467). *PCYT1A* exons were PCR-amplified from SMD-CRD patient genomic DNA with Accuprime Taq polymerase (Invitrogen, Cat#12339-016) and standard thermal cycling conditions using custom primer sequences.

***PCYT1A* cDNA construct generation**

RNA was extracted from *PCYT1A*-wild-type human fibroblasts using the RNeasy Mini Kit (Qiagen, Cat#74104), reverse-transcribed according to manufacturer protocol (SuperScriptIII One-Step RT-PCR system with Platinum Taq DNA Polymerase;Invitrogen, Cat# 12574-018), and cloned into the pcDNA3 mammalian expression vector (Invitrogen, V790-20) using primers designed to amplify full-length *PCYT1A* cDNA (based on NCBI transcript NM_005017.2) and attach restriction enzyme sites for subsequent cloning (forward primer: 5'-

TACGTAAGCTTAGCGCCACCTCAGAAGATAA-3', reverse primer: 5'-CTGAGCTCGAGTTGGGGTCACAATTTGGAAT-3'). Single base-pair SMD-CRD missense variants were then introduced using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, Cat # 200521). All plasmids were sequence-verified prior to use.

Plasmid transfection

Transfections were conducted using Lipofectamine 2000 as prescribed by manufacturer protocol (Invitrogen, Cat#11668-019) with ratios of 1.6 μg total plasmid DNA: 4 μL Lipofectamine for 12-well plate transfections, or ratios of 4.0 μg total plasmid DNA: 10 μL Lipofectamine for 6-well plate transfections. For lipid droplet rescue experiments, plasmid cDNAs harboring wild-type or SMD-CRD patient variants in *PCYT1A* were transfected into *PCYT1A*-null HEK293 cells and 24 hours post-transfection, cells were incubated with 1 mM oleate medium and further processed for imaging, as described below.

Protein extraction and western blotting

Upon reaching ~80% confluency, cells were quickly washed, scraped into ice-cold 1xPBS supplemented with protease inhibitors (Sigma-Aldrich, P8340-5ML), and centrifuged for 5 minutes at 1,000 x g. Cells were then resuspended in RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) with protease inhibitors (Sigma-Aldrich, P8340-5ML), vortexed briefly, and lysed at 4° C with rotation and periodic low-speed vortexing. Cell debris were pelleted via centrifugation at 12,000 x g for 15 minutes, and the supernatant was isolated for subsequent analysis. We measured total protein concentrations of cell lysates with the

BCA protein assay kit (Pierce, 23225) and diluted 40 μg of each protein with XT sample buffer (Bio-Rad, 1610791) and XT reducing agent (Bio-Rad, 1610792) as prescribed by company protocols. Samples were denatured by boiling for 5 minutes, then cooled and run on 4-12% Bis-Tris Criterion XT precast gels (Bio-Rad, 3450123) with XT MOPS Running Buffer (Bio-Rad, 1610788). Transfer was conducted onto Immun-Blot PVDF membranes (Bio-Rad, 162-0177) at 4° C with 100 V for 1 hour in 1 x Tris/Glycine Buffer (Bio-Rad, 1610734) and 20% methanol (Fisher Scientific, A41220). Blots were blocked for 1 hour at room temperature in 5% nonfat dried milk in TBST (0.5% Tween-20, 137 mM NaCl, 200 mM Tris, pH 7.5), then incubated in the appropriate primary antibodies (anti-β-actin antibody AC-15, ThermoFisher Scientific AM4302, 1:10,000 dilution; anti-CCTα, Abcam, ab109263, 1:1,000 dilution) diluted in 5% nonfat dried milk in TBST at 4° C overnight. Membranes were washed 3 x 10 minutes in TBST, incubated for 1 hour at room temperature in the appropriate secondary antibodies diluted 1:10,000 in 5% nonfat dried milk in TBST (goat anti-rabbit IgG-HRP, Santa Cruz Biotechnology sc-2004; goat anti-mouse IgG-HRP, Santa Cruz Biotechnology sc-2005). Membranes were washed 3 x 10 minutes in TBST, then incubated in ECL reagent (GE Healthcare Life Sciences, RPN2106) for 5 minutes and exposed to CL-X Posure Film (Thermo Scientific, 34091).

Phosphatidylcholine incorporation assay

To assess the effect of *PCYT1A* variants on the rate of phosphatidylcholine synthesis by the Kennedy pathway, we monitored continuous incorporation of [Methyl-^{3}H]-choline chloride (NEN Radiochemicals, NET109001MC) into phosphatidylcholine over time as described previously (Kitos, Drobnies, Ng, Wen, & Cornell, 2006). Wild-type or SMD-CRD patient fibroblast cell lines were cultured in 1xMEM with 10% FBS, 1% L-

glutamine, and non-essential amino acids in 60 mm dishes until ~80% confluency, and then counted using the Beckman Coulter Particle Counter Z1. Normal growth medium was aspirated, and cells were incubated in serum-free medium at 37° C with 5% CO_2 for 1 hour. We then added 5 μCi [^{3}H]-methyl-choline per dish and returned to the incubator, or quenched immediately for our 0 minute time point. After each time point was reached, cells were quickly rinsed in ice-cold 1xPBS, and radiolabel uptake was quenched by adding 1 mL of ice-cold methanol per dish. Cells were then placed on ice and scraped into prechilled glass tubes containing 1 mL chloroform, and residual cells were scraped with an additional 1 mL of methanol. We then added 0.75 mL H_20 per tube, mixed by vortexing at low speed, and added 1 mL additional chloroform followed by 1 mL of H_20 per tube. Cells were then vortexed briefly to mix, followed by centrifugation for 5 minutes at 1000 RPM to form a biphasic mixture. The lower organic phase (containing lipids) was then transferred to a scintillation vial, evaporated under nitrogen gas (Organomation Associates, Inc., Berlin, MA, USA) and resuspended in 10 mL Insta-Gel Plus scintillation fluid (Perkin Elmer, 6013399), followed by counting with a Beckman LS6500 scintillation counter. Previous thin layer chromatography analyses have shown that >90% of counts in the organic phase following up to 2 hours incubation are attributable to phosphatidylcholine (M. N. P. Ng, Kitos, & Cornell, 2004), so counts in this phase were used as a proxy for radiolabeled phosphatidylcholine levels. All sample counts were corrected for background counts, obtained using a blank scintillation vial.

Genome editing

Genome editing was performed as described previously, with slight modification (Moyer & Holland, 2015). Guide RNAs against target genomic sequences from human or

mouse *PCYT1A* (RefSeq NC_000003.11 or NC_000082.5, respectively) were designed using the CRISPR MIT portal (Hsu et al., 2013), modified into oligonucleotides for cloning purposes, and cloned according to the submitter's laboratory protocol into the pSpCas9(BB)-2A-Puro (PX459) V2.0 plasmid (Addgene, Cat #62988), which was a gift from Feng Zhang's laboratory (Ran et al., 2013). By combining two guide RNAs targeting the beginning and end of the genomic sequence of either human or mouse *PCYT1A*, we generated ~31 kb genomic deletions in either human (HEK293 cells) or mouse (ATDC5 cells) *PCYT1A*, effectively excising the majority of the coding sequence. Cells were seeded for transfection at ~2 x 10^5 cells/well in six-well plates, grown to ~80% confluency, and transfected using Lipofectamine 2000 as prescribed by manufacturer protocol (Invitrogen, 11668-019), with equal DNA amounts of each guide RNA-encoding plasmid (2 μg each plasmid DNA: 10 μl Lipofectamine). Next, we treated cells with puromycin-containing medium at either 2 μg/mL (HEK293 cells) or 4 μg/mL (ATDC5 cells) 24 hours post-transfection. Medium was replaced daily for 2-3 days, until cells in untransfected control wells died. Single cells were then isolated by serial dilution in puromycin-free medium, expanded into isogenic clones, subjected to DNA extraction, and screened for both wild-type and deletion-containing genomic DNA by PCR amplification. Clones which contained the *PCYT1A* deletion but not wild-type genomic DNA were genotyped using Sanger sequencing and tested for CCTα protein levels by western blot analysis.

Cell proliferation assays

Cell proliferation was measured using the Cell Counting Kit-8 (Dojindo Molecular Technologies, CK04-05), as prescribed by manufacturer protocol. Cells were counted

using the Beckman Coulter Particle Counter Z1, plated at 1000 cells per well in 96-well plates, and cultured for the respective amounts of time indicated in our growth curve. Growth was measured by incubating with CCK-8 solution for 3 hours, followed by absorbance reading at 450 nm with the Biotek Synergy 2 plate reader and Gen5 software v2.01.14.

Oleate incubation for lipid droplet and nuclear lamina translocation studies

In order to induce lipid droplet formation, oleic acid: BSA complexes were prepared for addition to cell culture medium. First, we prepared a 100 mM sodium oleate solution (Sigma-Aldrich, cat#O3880-1G) in distilled water and sonicated on ice (Branson Sonifier 250 with tapered microtip, Fisher Scientific, Cat#22-309782) under a constant duty cycle and output level of 2 for approximately 2 minutes, or until solution reached clarity. We then mixed this solution with a 200 mg/ml fatty acid-free BSA (Sigma-Aldrich, A8806-5G) in PBS and briefly sonicated again until solution was clear. This generated a final stock of 100 mg/mL BSA with 10 mM oleate, which we diluted 10-fold in serum-free medium (1 x MEM supplemented with non-essential amino acids and 1% L-glutamine) for a 1 mM working oleate solution. Cells were incubated in 1 mM oleate medium for various timepoints prior to processing for immunofluorescence or lipid droplet staining.

Cell staining and immunofluorescence

Cell culture was performed using the conditions described above in 12-well plates containing sterile 18-mm glass cover slips (VWR, 48380-046). Cells were fixed for 10 minutes in 4% paraformaldehyde (Sigma-Aldrich, P6148-500G), permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, T-8532) in 1 x PBS for 10 min, and blocked in 1% BSA

(Sigma-Aldrich, A-9647) in 1 x PBS for 1 hour, all at room temperature. Cells were then processed for immunofluoresence or lipid droplet staining.

For immunofluorescence experiments, cells were incubated in primary antibody (anti-CCTα, Abcam, ab109263) diluted 1:200 in 1% BSA in PBS overnight at 4°C. Cells were then incubated in Alexa Fluor 488 or 555 secondary antibodies (Thermo Fisher Scientific, A-21428 or A-11008) diluted 1:300 in 1% BSA in PBS for one hour at room temperature. For lipid droplet staining, cells were incubated for one hour in BODIPY 493/503 dye (ThermoFisher Scientific, Cat#D-3922, stored as 1 mg/mL stock in 100% ethanol at -20°C) diluted in 1:500 in 1 x PBS.

Cell nuclei were counterstained with DAPI (Life Technologies, D1306) diluted to 300 nM in 1 x PBS for 10 minutes at room temperature, followed by mounting with ProLong Gold Antifade Mountant (Thermo Fisher Scientific, P36930) on glass slides (Fisher Scientific, 12-550-15). Images were obtained using the Zeiss LSM 510 Meta Confocal Microscope under 63x oil magnification.

Lipid Droplet quantification

Lipid droplet sizes and numbers were quantified as described previously (Aitchison et al., 2015). Using Image J software (v1.47), we converted images to 8-bit, adjusted image threshold, and used the "analyze particle" command with a particle size distribution of 20 to infinity pixels^2 set to exclude on edges.

Alcian blue staining

Alcian blue was performed as previously described (Lu et al., 2016), with slight modification. Upon reaching ~80% confluency, cells were rinsed in 1xPBS, fixed with 4% paraformaldehyde for 10 minutes at room temperature, and then incubated in 1% Alcian

Blue 8GX (Sigma-Aldrich, A5268-10G) pH 1.0 at 4° C overnight. After briefly washing in 1xPBS, cells were lysed in 1% SDS for one hour and absorbance was measured at 605 nm using the Biotek Synergy 2 plate reader with Gen5 software v2.01.14.

Lipid standards for mass spectrometry

The following lipid standards were obtained from Avanti Polar Lipids (Alabaster, AL): 1,2-dilauroyl-*sn*-glycero-3-phosphate (sodium salt) (PA 12:0/12:0, PA C12), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (PC 12:0/12:0, PC C12), 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine (PE 12:0/12:0, PE C12), 1,2-dilauroyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt) (PG 12:0/12:0, PG C12), 1,2-dilauroyl-*sn*-glycero-3-phospho-L-serine (sodium salt) (PS 12:0/12:0, PS C12), cholesteryl-d7 palmitate (Cholesterol-d7 ester 16:0, Cholesterol ester d7), N-lauroyl-D-*erythro*-sphingosine (C12 Ceramide d18:1/12:0, Cer C12), N-heptadecanoyl-D-erythro-sphingosine (C17 Ceramide d18:1/17:0, Cer C17), N-lauroyl-D-erythro-sphingosylphosphorylcholine (12:0 SM d18:1/12:0 SM C12), 1,3-dihexadecanoyl glycerol (d5) [1,3-16:0 DG (d5), DG d5], 1,3(d5)-dihexadecanoyl-2-octadecanoyl-glycerol [TG d5-(16:0/18:0/16:0), TG d5], D-galactosyl-ß-1,1' N-lauroyl-D-*erythro*-sphingosine [C12 Galactosyl(ß) Ceramide (d18:1/12:0), GlcCer C12], D-lactosyl-ß-1,1' N-dodecanoyl-D-*erythro*-sphingosine [Lactosyl (ß) C12 Ceramide, LacCer C12]. We purchased APCI positive calibration solution from AB Sciex (Cat. #4460131; Concord, Ontario, Canada).

Stock solutions of PC C12 (12.5 mg/mL), PS C12 (5 mg/mL), and SM C12 (5 mg/mL) were dissolved in methanol. Stock solutions of Cer C12 (2.5 mg/mL), Cer C17 (2.5 mg/mL), PE C12 (6.25 mg/mL), PA C12 (3.125 mg/mL), PG C12 (6.25 mg/mL), DG d5

(0.25 mg/mL), TG d5 (0.25 mg/mL), LacCer C12 (0.625 mg/mL) and GlcCer C12 (1.25 mg/mL) were dissolved in dichloromethane/methanol 1:1 (v/v). Finally, cholesterol ester d7 was dissolved in dichloromethane. All solutions were stored at -20°C until use. Ultrapure water (resistivity > 18 MΩ cm) was used for mass spectrometry experiments.

Cell culture and lipid extraction for mass spectrometry

For mass spectrometry experiments, we measured various lipid classes in *PCYT1A* wild-type versus *PCYT1A*-null HEK293 and ATDC5 cells, or in cultured dermal fibroblasts obtained from *PCYT1A* wild-type controls or SMD-CRD patients homozygous for the p.Ser323Argfs*38 variant in *PCYT1A*. All cells were cultured at 37° C under 5% CO_2 and passaged using 1 x trypsin-EDTA (Gibco, Cat. #15400-054). HEK293 and fibroblast cell lines were cultured in 1 x MEM (Gibco, Cat. #11430-030) supplemented with non-essential amino acids (Gibco, Cat. #11140-050), 1% L-glutamine (Gibco, Cat. #25030-149), and 10% fetal bovine serum (Gemini Biosciences, Cat. #100-106). ATDC5 cells were maintained in DMEM/F12 50/50 (Corning Cellgro, Cat. #15-090-CV) with 1% L-glutamine (Gibco, Cat. #25030-149), and 5% fetal bovine serum (Gemini Biosciences, Cat. #100-106), and 1% penicillin-streptomycin (Gibco, Cat. #15140-122). Upon reaching ~80% confluency, cells were scraped in ultrapure water, pelleted at 1000 x g, resuspended in ultrapure water, and subjected to total protein concentration measurements using the BCA protein assay kit (Cat. #23225, Pierce). Cell extracts were stored at -80°C until further processing.

We isolated lipids from cell extracts using a modified Bligh-Dyer procedure. 200 µg protein from each cell suspension was transferred into 1.5 mL plastic centrifuge tubes and adjusted to 200 µL total volume with ddH_2O. We then sonicated cell solutions to form

homogenates by applying short bursts with a sonic dismembrator (Fisher Scientific, Waltham, MA). Homogenates were transferred to glass tubes and gently mixed with 800 µL of ddH_2O, for a final volume of 1 mL. We next formed a monolayer by adding 2.9 mL methanol/dichloromethane (2:0.9, v/v) containing internal standards for 12 lipid classes to each sample. We then added 1 mL of ddH_2O and 0.9 mL dichloromethane to obtain a biphasic mixture, which was incubated at 4^{o}C for 30 min and centrifuged at 4 ^{o}C for 10 min at 3000 x g to separate organic and aqueous phases. 1 mL of the organic phase was transferred to a 2 mL glass vial and stored at -20^{o}C until use. We finally dried 0.5 mL of the organic layer extract using a nitrogen evaporator (Organomation Associates, Inc., Berlin, MA, USA) and re-suspended in 135 µL of running solvent (dichloromethane:methanol (1:1) with 5 mM ammonium acetate) containing 5 mg/mL of ceramide (C17:0) as an internal standard. All solvents used were HPLC grade.

Untargeted lipid analysis by MS/MS^{ALL}

Mass spectrometry analyses were performed via MS/MS^{ALL} on a TripleTOF^{TM} 5600 mass spectrometer (AB SCIEX, Redwood City, CA). We applied 50 µL of each lipid extraction to the system by direct infusion using a DuoSpray electrospray ionization source and autosampler (Shimazu, Canby, OR) at a flow rate of 7 µL/min. Samples were run in duplicate in the positive ion mode. A dichloromethane: methanol solution (1:1, v/v) with 5 mM ammonium acetate was used as the running solvent. We used a mass resolution of ~30 000 for TOF MS scans and ~15 000 for product ion scans in high sensitivity mode, and automatically calibrated using APCI positive calibration solution delivered via calibration delivery system (AB Sciex) after every 10 sample injections. We used the following source parameters: ion source gases 15 psi (GSI), 20 psi (GS2),

curtain gas 30 psi, temperature 150^{o}C, positive ion spray voltage +5200 V, declustering potential of 80V and collision energy of 10V. Initial TOF MS scanning provided an overview of total lipid content at an accumulation time of 5s. Precursor ions were selected by sequential 1 Da mass steps from 200.050 to 1200.050 m/z. Analytes in each 1 Da step were introduced into the collision chamber, and fragments were identified by TOF with a scan range of 100-1500 m/z (accumulation time of 300 ms). The collision energy for each MS/MS step was 40eV. In addition, a pooled sample containing extracts from all groups was run 8 times for lipid identification and subsequent selection of targeted lipids. All data was acquired using Analyst 1.7 TF (AB SCIEX, Concord, ON, Canada).

Mass spectrometry data processing and analysis

The TOF MS and MS/MS^{ALL} data obtained from individual samples and pooled samples were aligned post-acquisition to internal standards using Analyst 1.7 TF. We conducted broad assignment and identification of lipids on the aligned data from pooled samples using LipidView^{TM} software (v1.3 Beta, AB SCIEX, Concord, ON, Canada). To reduce false-positive identifications due to instrumental noise, lipids whose MS/MS fragment peaks appeared in at least 7 of 8 pooled runs were considered for further analysis. We then calculated the coefficient of variation (CV) of the fragment peak intensity for each lipid relative to the internal standard for this class of lipid obtained over multiple runs. Lipids with CVs <20% were considered reliable and used as targeted lipids in order to identify lipids of these classes in experimental samples using LipidView^{TM}. We calculated CV values and generated targeted lipid lists using a custom in-house Matlab program. MultiQuant software (version 3.0, AB SCIEX, Concord, ON, Canada)

was used to identify lipids in experimental samples by comparing to the targeted lipid list described above. Peak intensities were corrected using internal standards for each class of lipid, and we averaged duplicates within each sample for further analysis. To ensure that data could be statistically analyzed, any zero-value intensities were replaced by 0.000001. We then proceeded with additional data analysis using R 3.4.2 (Bioconductor). Data were log-transformed, z-score normalized, and exponentially transformed so that all values were positive. In order to compare each lipid between *PCYT1A* wild-type and variant cell lines of each group (ATDC5, HEK293, or fibroblast cell lines), we used Welch's two-sample *t*-test.

Statistical procedures

All statistical analyses were performed using R 3.2.4 (Bioconductor). Multiple group comparisons were done using Tukey's Honest Significant Difference test. No outliers were removed for statistical analyses.

Results

Measurement of CCTα steady state levels and PC incorporation rates

Western blot analysis of CCTα steady state levels in cultured fibroblasts of five SMD-CRD patients (4 probands and one affected sibling) revealed reduced CCTα steady state levels in all patient cell lines as compared to wild-type controls, with the level of reduction varying according to patient genotype (Figure 3A). Fibroblasts homozygous for the p.Ala99Val variant had the mildest reductions in CCTα steady state levels (~75% wild-type levels), followed by p.Glu129Lys homozygotes (~30% wild-type levels). Fibroblasts homozygous for a 1-bp frameshifting insertion (p.Ser323Argfs*38) had only

~10% of wild-type CCTα levels, and similar levels were observed in cells compound heterozygous for the p.Ser323Argfs*38/p.Ser114Thr variants.

To assess PC synthesis, we measured continuous incorporation of [^{3}H]-choline into PC in intact fibroblasts over 2 hours. Consistent with the results of our western blotting experiments, p.Ala99Val homozygotes had the mildest impairments in PC incorporation (54% of wild-type), whereas homozygotes for the p.Ser323Argfs*38 or p.Glu129Lys alleles had only 22% of wild-type incorporation levels, and compound heterozygotes for the p.Ser323Argfs*38/p.Ser114Thr alleles had 33% of wild-type incorporation.

CCTα membrane translocation in putative cell models

In order to assess the utility of several cell models for studying CCTα variants, we first analyzed CCTα localization in response to oleate treatment. Previous studies have shown that oleate stimulation can induce CCTα translocation to the nuclear lamina in CHO cells and in HEK293 cells, although results are variable depending on the report and the cell line tested (Aitchison et al., 2015; Gehrig et al., 2008; Lagace & Ridgway, 2005). Under our experimental conditions, oleate failed to stimulate translocation of CCTα to membranes in wild-type fibroblasts or in CHO cells, but did induce membrane translocation in wild-type HEK293 cells. Based on this finding, we decided to move forward with additional cellular modeling in HEK293 cells to examine consequences of *PCYT1A* variants on cellular phenotypes.

Lipid droplet analyses

In cells from Drosophila, mice, and rats, deficiency of CCTα orthologs results in formation of fewer and larger lipid droplets than in control cells in response to oleate exposure (Aitchison et al., 2015; Guo et al., 2008; Krahmer et al., 2011). Based on this,

we hypothesized that SMD-CRD patient fibroblast cells would also develop fewer and larger lipid droplets in response to oleate treatment. However, this was not the case. The sizes and numbers of lipid droplets in both wild-type and SMD-CRD patient fibroblast cell lines were highly heterogeneous, and SMD-CRD patient fibroblasts (p.Ser323Argfs*38 homozygotes) failed to show significant differences in lipid droplet numbers or sizes as compared to control cells (Fig. 4A and 4C). Interestingly, however, *PCYT1A*-null HEK293 cells generated fewer and larger lipid droplets than their wild-type counterparts in response to oleate loading (Fig. 4B and 4D). Transfection with wild-type, p.Ala99Val, and p.Tyr240His *PCYT1A* cDNAs, but not empty vector cDNA, rescued lipid droplet phenotypes in *PCYT1A*-null cells (Fig. 4E).

Proliferation studies in HEK293 cells

Interestingly, *PCYT1A*-null HEK293 cells proliferated at normal rates as compared to wild-type cells (Fig. 5A). We hypothesized that this might be attributable to exogenous serum lipid uptake in *PCYT1A*-null cells, so we next determined the effects of culturing cells in delipidized serum. Both wild-type and *PCYT1A*-null HEK293 cells grew slower in delipidized serum as compared to normal serum, but surprisingly *PCYT1A*-null HEK293 cells actually grew more effectively than their wild-type counterparts in delipidized serum (Fig. 5A).

Effects of *Pcyt1a* knockout on chondrocyte proliferation and differentiation

To evaluate the utility of ATDC5 pre-chondrocytes as a model system, we first assessed their steady-state CCTα levels at baseline and over the course of 21 days of chondrocyte differentiation by western blot analysis. CCTα protein was expressed robustly in ATDC5 cells prior to chondrocyte differentiation and maintained stable

expression over the 21-day differentiation timecourse (Fig. 6A). Next, we generated *Pcyt1a*-null ATDC5 cell lines using genome editing and assessed the consequences of *Pcyt1a* loss-of-function on chondrocyte proliferation and differentiation. Similar to our observations in HEK293 cells, *Pcyt1a*-null ATDC5 cells proliferated at normal rates as compared to their wild-type counterparts (Fig. 5B). However, *Pcyt1a*-null ATDC5 cells actually differentiated into chondrocytes more quickly than their wild-type counterparts (Fig. 6B).

Untargeted lipidomic studies

To assess global changes in lipid metabolism in SMD-CRD patient-derived skin fibroblasts (p.Ser323Argfs*38 homozygotes) and in *PCYT1A*-null HEK293 or ATDC5 cells as compared to their wild-type counterparts, we conducted untargeted mass spectrometry on ~1000 distinct lipids. In total, the levels of ~75-200 lipids were significantly increased or decreased in the *PCYT1A*-null or SMD-CRD condition for each cell line tested, and the specific lipids altered were largely variable depending on the cell type tested, with one exception. The levels of several individual LPCs (Fig. 7A) were significantly decreased in *PCYT1A*-null HEK293 cells (LPCs 16:1, 18:1, 18:2, 20:0, 20:3, 20:4, 20:5, 22:5, and 22:6) and in SMD-CRD patient fibroblasts (LPCs 16:0, 16:1, 16:3, 18:1, 18:2, 20:1, 20:4, 22:4, and 22:5). Notably, levels of five identical LPCs (LPCs 16:1, 18:1, 18:2, 20:4, and 22:5) were reduced in both *PCYT1A*-null HEK293 cells and in SMD-CRD patient fibroblast cell lines as compared to controls.

We first examined whether the proportions of specific lipid classes in each cell line was altered in *PCYT1A*-null or SMD-CRD conditions as compared to wild-type controls. We grouped individual lipids in each cell line into the following categories: cholesterol

ester, ceramide, desmosteryl ester, diacylglycerol, galactosylceramide, lactosylceramide, lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylglycerol, lysophosphatidylserine, monoakyldiacylglyceride, phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, sphingomyelin, or triacylglyceride. Some lipid classes were not represented in all cell lines tested. In *PCYT1A*-null ATDC5 cells as compared to their wild-type counterparts, we observed significant decreases in the overall proportions of lysophosphatidylcholines (4-fold) and lysophosphatidylethanolamines (2-fold). In SMD-CRD patient cells homozygous for the p.Ser323Argfs*38 variant as compared to wild-type controls, we observed significant increases in the overall proportions of diacylglycerol (1.9-fold) and phosphatidylethanolamine (1.6-fold). Finally, in *PCYT1A*-null HEK293 cells as compared to their wild-type counterparts, we observed significant decreases in the proportions of lysophosphatidylcholine (2-fold) and phosphatidylcholine (2.5-fold), as well as significantly increased proportions of galactosylceramide (1.9-fold), lactosylceramide (2-fold), and lysophosphatidylserine (2.5-fold).

Next, we analyzed whether the ratios of specific lipid classes were altered in *PCYT1A*-null or SMD-CRD patient cells as compared to wild-type controls for each cell type. First, we found that sphingomyelin:ceramide (SM:Cer) ratios were not significantly altered in any of the *PCYT1A*-null or SMD-CRD patient cell lines as compared to their wild-type counterparts (data not shown). However, phosphatidylcholine:phosphatidylethanolamine (PC:PE) ratios were reduced significantly by 2-3 fold in *PCYT1A*-null HEK293 cells and in SMD-CRD patient fibroblast cell lines as compared to their wild-type counterparts (Fig. 7B).

Discussion

Through western blot analyses PC incorporation assays, we determined that all SMD-CRD patient-derived dermal fibroblasts tested were hypomorphic to varying degrees, ranging from 10-75% of wild-type steady state CCTα protein levels and 22-54% of wild-type PC incorporation. Surprisingly, the level of reduction in steady-state CCTα or PC incorporation does not appear to correlate with the severity of patients' phenotypes. Fibroblasts homozygous for p.Ala99Val had the mildest biochemical phenotype of all variants tested, both in their CCTα steady state levels (75% of wild-type) and PC incorporation (54% of wild-type). Because the p.Ala99Val allele decreased PC incorporation more severely than steady-state expression, we conclude that this allele reduces the catalytic activity of CCTα independently of its effects on protein production or stability. This conclusion is further supported by the location of the affected amino acid in the crystal structure of the orthologous rat CCTα protein: the alanine residue falls adjacent to a glutamine which is involved in CDP coordination, and hence is important for catalytic activity (J. Lee et al., 2009).

Other alleles reduced steady state levels of CCTα and PC incorporation much more severely: for instance, fibroblasts homozygous for the p.Ser323Argfs*38 allele had only 10% of the steady state CCTα and 22% of the PC incorporation levels of wild-type fibroblasts. We did not expect this allele to result in complete loss of CCTα expression, since the generated termination codon is C-terminal to the penultimate exon junction and therefore is not predicted to be subject to nonsense-mediated mRNA decay. However, the strong decrease in steady state CCTα coupled with less severe reductions in PC incorporation suggest that this allele results in an unstable protein with normal or perhaps

increased catalytic activity resulting from its modified C-terminus, which typically regulates activation of the enzyme.

Compound heterozygous fibroblasts for p.Ser323Argfs*38/p.Ser114Thr alleles had only 14% of wild-type steady state CCTα but 33% of wild-type PC incorporation. The protein levels in this cell line were only slightly higher than those of p.Ser323Argfs*38 homozygotes, indicating that the p.Ser114Thr allele has similarly adverse consequences on protein production or stability, at least when in combination with the p.Ser323Argfs*38 allele. However, similar to p.Ser323Argfs*38 homozygotes, the residual CCTα protein in compound heterozygotes seems to have normal or even increased activity as compared to wild-type protein. Finally, fibroblasts homozygous for p.Glu129Lys had ~30% of wild-type steady-state CCTα levels and similar levels of PC incorporation (22% of wild-type), indicating that this variant protein is unstable and has normal or only mildly reduced CCTα activity. This glutamic acid residue falls in an α-helix at the CCTα dimerization interface which is part of a larger series of α-helices and β-strands comprising a Rossman fold domain (J. Lee et al., 2009). It is possible that this variant destabilizes CCTα by decreasing its capacity to dimerize.

Contrary to our expectations based on prior studies in Drosophila, mouse, and rat cell lines (Aitchison et al., 2015; Guo et al., 2008; Krahmer et al., 2011), SMD-CRD patient fibroblasts (p.Ser323Argfs*38 homozygotes) failed to show significant differences in lipid droplet numbers or sizes as compared to controls, indicating that oleate loading does not induce an abnormal lipid droplet phenotype in cells with this genotype. Of note, p.Ser323Argfs*38 homozygous fibroblasts had the most severe reductions of all SMD-CRD patient cells tested in both steady-state levels of CCTα (~10% wild-type levels) and

in phosphatidylcholine incorporation (~22% wild-type levels), so the absence of a lipid droplet phenotype in our experiments was surprising. This may be because the mechanism for regulating lipid droplet sizes and numbers is not conserved in human fibroblasts. Alternatively, it is possible that complete ablation of CCTα protein expression is required for lipid droplet phenotypes to manifest; indeed, SMD-CRD patient fibroblasts retained some residual CCTα expression and activity, which could be sufficient to normalize lipid droplet formation. Consistent with previous publications, *PCYT1A*-null HEK293 cells generated fewer and larger lipid droplets than did wild-type HEK293 cells in response to oleate loading, suggesting again that CCTα-mediated lipid droplet regulation may be cell-type specific in cultured human cell lines, or that complete ablation of *PCYT1A* expression may be required in order for lipid droplet phenotypes to manifest. Transfection with wild-type, p.Ala99Val, and p.Tyr240His *PCYT1A* cDNAs, but not empty vector cDNA, rescued lipid droplet phenotypes in *PCYT1A*-null cells. This may indicate that these variants in the catalytic or membrane-binding domains of CCTα provide ample enzyme activity and PC production to generate lipid droplets normally; this interpretation would be consistent with our findings in SMD-CRD patient fibroblasts, in which even small residual amounts of functional CCTα are sufficient to regulate lipid droplet formation normally. However, we also recognize that cDNA expression was driven by a CMV promoter in our transfection studies, which may simply normalize lipid droplet phenotypes by *PCYT1A* overexpression.

Given that *Pcyt1a*-null mice are embryonic lethal and CHO MT-58 cells undergo apoptosis (Cui et al., 1996; Sweitzer & Kent, 1994; L. Wang et al., 2005), we were intrigued that *PCYT1A*-null HEK293 and ATDC5 cells were grossly morphologically

normal and proliferated at normal rates as compared to their wild-type counterparts. However, these results are consistent with findings that *Pcyt1a*-null mouse peritoneal macrophages grow normally under standard cell culture conditions due to compensatory upregulation of a specific isoform of *Pcyt1b* (D. Zhang et al., 2000). Interestingly, however, these *Pcyt1a*-null mouse macrophages had increased susceptibility to cell death upon loading with free cholesterol, suggesting that additional stressors may be required to evoke cell death in CCTα-deficient cell lines. Furthermore, we found that *PCYT1A*-null HEK293 cells proliferated more quickly than their wild-type counterparts when cultured in delipidized serum. This suggests that growth rates are normalized independently of exogenous lipid uptake from serum in these cells, and that *PCYT1A*-null HEK293 cells may have a growth advantage over their wild-type counterparts when grown in conditions devoid of exogenous lipids, perhaps by upregulation of compensatory pathways for PC production.

Using untargeted mass spectrometry, we were able to investigate changes in lipid species apart from those directly implicated in the Kennedy pathway. We found that a broad array of lipids are increased or decreased in *PCYT1A*-null HEK293 or ATDC5 cells or SMD-CRD patient fibroblast cell lines as compared to controls, and the altered lipids were often cell-type specific. However, we noticed a few trends that replicated in more than one of the three cell lines tested. First, total PC:PE ratios were significantly decreased in *PCYT1A*-null HEK293 and in SMD-CRD patient fibroblast cell lines as compared to controls. Decreased PC:PE ratios have previously been associated with the loss of membrane integrity and decreases in membrane potential in certain cell types (Z.

Li et al., 2006), so it is possible that decreased ratios of these lipids have broad adverse consequences.

In addition, we found that several LPCs were significantly decreased in both *PCYT1A*-null HEK293 and in SMD-CRD patient fibroblast cell lines as compared to the corresponding control cell lines. Interestingly, five of these decreased LPCs had the same acyl chain compositions in both cell lines: LPC 16:1, 18:1, 18:2, 20:4, and 22:5. Decreased LPCs could be attributable either to increased conversion of LPC into PC by LPCAT or to decreased conversion of PC into LPC by PLA_2. Previous studies in COS cells and in CHO cells have shown that CCTα overexpression increases LPC levels by promoting PLA_2-mediated PC catabolism (Walkey, Kalmar, & Cornell, 1994; Barbour, Kapur, & Deal, 1999), so it is possible that decreased activity by this pathway is also responsible for decreases in LPC in our cell lines. However, recent studies have also demonstrated increased *Lpcat4* mRNA expression and activity toward LPC acyl chains 18:1, 18:2, 20:4, and 22:6 during the late stages of chondrocyte differentiation in ATDC5 cells (Tabe et al., 2017). Notably, LPCs with three of these four acyl chains (18:1, 18:2, and 20:4) were decreased in SMD-CRD patient fibroblast cell lines, and all four were decreased in *PCYT1A*-null HEK293 cells as compared to wild-type controls in our study. The mean levels of these and other individual LPCs were also decreased in *Pcyt1a*-null as compared to wild-type ATDC5 cells, but none of these decreases approached statistical significance. This may be due in part to the small sample size of ATDC5 cells measured in our study, so we suggest further investigation of LPCs in this cell type for future studies. Observed decreases in individual LPC species were also consistent with

decreases in overall LPC content in both *PCYT1A*-null HEK293 cells and ATDC5 cells as compared to wild-type controls.

Decreased LPCAT expression or activity may also be supported by our finding that *Pcyt1a*-null ATDC5 cells have increased rates of chondrocyte differentiation as compared to wild-type controls. *Lpcat4* knockdown has been associated with decreased rates of chondrocyte differentiation in ATDC5 cells (Tabe et al., 2017), so it is possible that compensatory upregulation of *Lpcat4* expression or activity contributes to the increased rate of chondrocyte differentiation in *Pcyt1a*-null ATDC5 cells. Changes in chondrocyte differentiation rates have also been observed in previous studies of other skeletal dysplasias. For instance, ATDC5 cells expressing the variant responsible for achondroplasia have slowed rates of chondrocyte differentiation, whereas mice null for parathyroid hormone-related protein have accelerated rates of chondrocyte differentiation (Lanske et al., 1996; Matsushita et al., 2013). Our results suggest that SMD-CRD may be another phenotype for which perturbed rates of chondrocyte differentiation contribute to skeletal dysplasia.

The overall proportions of lipids represented in each SMD-CRD variant or *PCYT1A*-null cell line were also altered, but the nature of the alterations varied among cell types. As mentioned previously, the only lipid whose overall cellular content was decreased in two cell types was LPC, which was present in lower proportions in *PCYT1A*-null HEK293 and ATDC5 cell lines as compared to corresponding wild-type controls. In *Pcyt1a*-null ATDC5 cells, we also observed decreases in overall LPE content, suggesting potential upregulation of LPE acyltransferase (LPEAT) or downregulation of PLA_2. In *PCYT1A*-null HEK293 cells, we observed decreased PC content, suggesting that

compensatory pathways for PC production may not be as robust in this cell type as in others. *PCYT1A*-null HEK293 cells also had increased content of LPS, galactosylceramide, and lactosylceramide. However, the reasons or implications for increased levels of these lipids are unclear. Both galactosylceramide and lactosylceramide are formed from ceramide, which can combine with PC to form sphingomyelin in a reaction catalyzed by sphingomyelin synthase. Perhaps decreased PC production by the Kennedy pathway promotes downregulation of sphingomyelin synthase in an attempt to maintain cellular PC, and in turn promotes increases in other ceramide metabolic pathways such as those required for galactosylceramide and lactosylceramide production. In homozygous p.Ser323Argfs*38 fibroblasts, the proportions of DAG and PE were increased as compared to wild-type fibroblasts. Increased proportions of DAG might be expected, given that SMD-CRD cells have decreased production of CDP-choline, which normally combines with DAG to form PC in the final step of the Kennedy pathway. However, increased proportions of PE were not expected *a priori* and suggest that in SMD-CRD fibroblasts, upregulation of the PEMT pathway is not likely a major means for compensatory PC production.

Overall, our results indicate that partial or complete loss of *PCYT1A* in ATDC5, HEK293, or fibroblast cell lines leads to decreased PC synthesis by the Kennedy pathway and cell-type specific secondary dysregulation of diverse additional lipid metabolic pathways, which manifests in lipid droplet phenotypes in HEK293 cells but not SMD-CRD patient fibroblasts. Our results also suggest involvement of *Pcyt1a* in chondrocyte differentiation but not proliferation or morphology. We suggest that a careful side-by-side assessment of all *PCYT1A* alleles implicated in SMD-CRD, isolated retinopathy, and

lipodystrophy in a range of cell types would be informative for dissecting further tissue-specific consequences of these alleles.

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Chapter 5 Figures

Figure 1. Diagram of all known SMD-CRD alleles to-date. Both previously described and novel SMD-CRD patient variants are mapped to their corresponding CCTα protein domains. Note that most variants are missense and fall within the catalytic domain. NLS-nuclear localization signal; M-membrane-binding domain; P-phosphorylation domain.

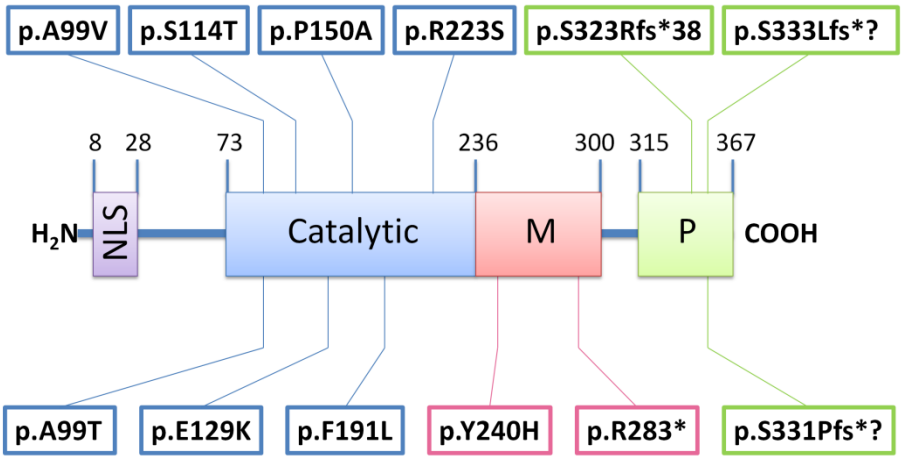


Figure 2. Kennedy pathway and related pathways for PC production. Choline (Cho) is taken in exogenously from the diet and transported across the plasma membrane into the cytosol, where it becomes phosphorylated by choline kinase to produce phosphocholine (P-Cho). Phosphocholine is converted into CDP-choline (CDP-Cho) in a reaction catalyzed by CTP:phosphocholine cytidylyltransferase (CCT), which is combined with diacylglycerol (DAG) to form phosphatidylcholine (PC). Phospholipase-A_2 (PLA_2) converts PC into lyso-phosphatidylcholine (LPC), which can be converted into PC once more in a reaction catalyzed by lysophosphatidylcholine acyltransferase (LPCAT). PC can also be derived from sequential methylation of phosphatidylethanolamine (PE) by phosphatidylethanolamine *N*-methyltransferase. The red rectangle indicates the location of the block in PC synthesis in SMD-CRD. Phosphatidylethanolamine can also be interconverted to lysophosphatidylethanolamine by phospholipase-A_2 (PLA_2) and LPE acyltransferase (LPEAT) enzymes.

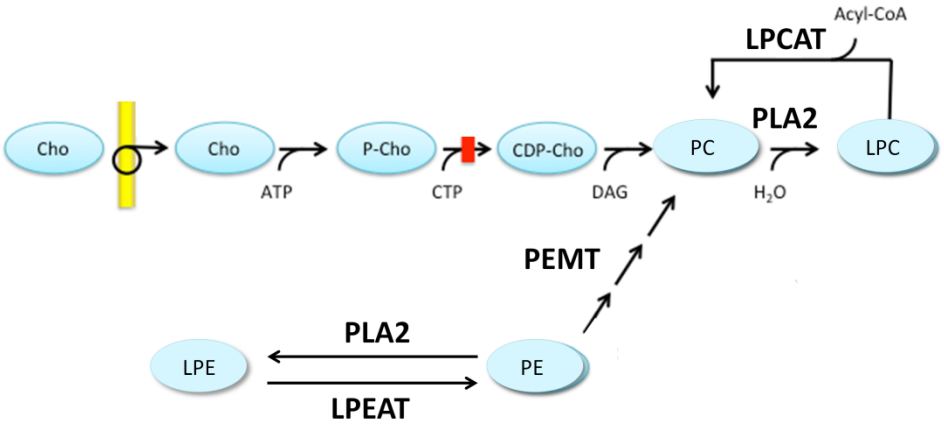


Figure 3. Western blot and PC incorporation assays of SMD-CRD patient fibroblasts. A) Representative immunoblot showing reduced protein expression of CCTα relative to loading control β-actin in cultured skin fibroblasts of five SMD-CRD patients as compared to wild-type controls, with the level of reduction varying according to patient genotype. Protein levels ranged from ~10-75% of wild-type CCTα. B) To assess PC synthesis, we measured continuous incorporation of [^{3}H]-choline into PC in intact fibroblasts over 2 hours. Cell lines again had variable PC incorporation levels, ranging from 22-54% of wild-type. Error bars represent standard deviation. n=3 biological replicates. Tukey's Honest Significance Test. *p<0.05.

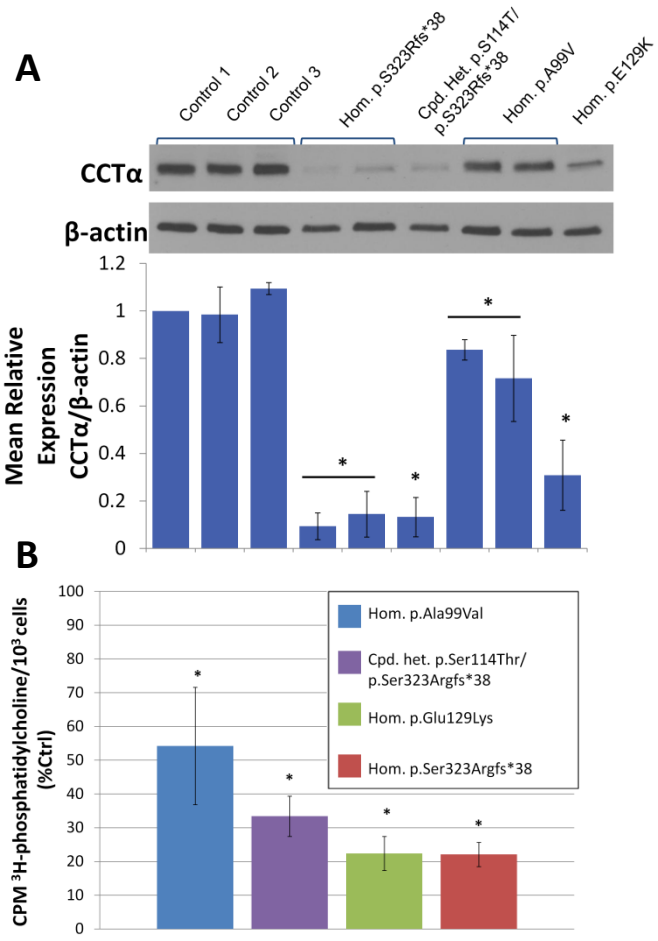


Figure 4. Lipid droplet experiments. Lipid droplet (LD) formation was induced by oleic acid loading and visualized with BODIPY staining. LD size and number were quantified using "analyze particle" command in ImageJ (v1.47). A and B) Neither LD size (p=0.43) nor number (p=0.87) differed between control and SMD-CRD patient fibroblasts. C and D) *PCYT1A*-null HEK293 cells have fewer and larger LDs than control cells. E) LD sizes and numbers are rescued by transfection with *PCYT1A* WT, p.Ala99Val or p.Tyr240His cDNAs but not corresponding empty vector controls. n=200 cells from 4 experimental trials. Error bars represent SD. Welch Two Sample t-test for panels A and C, and Tukey's Honest Significant Difference test for panel E. *p<0.05.

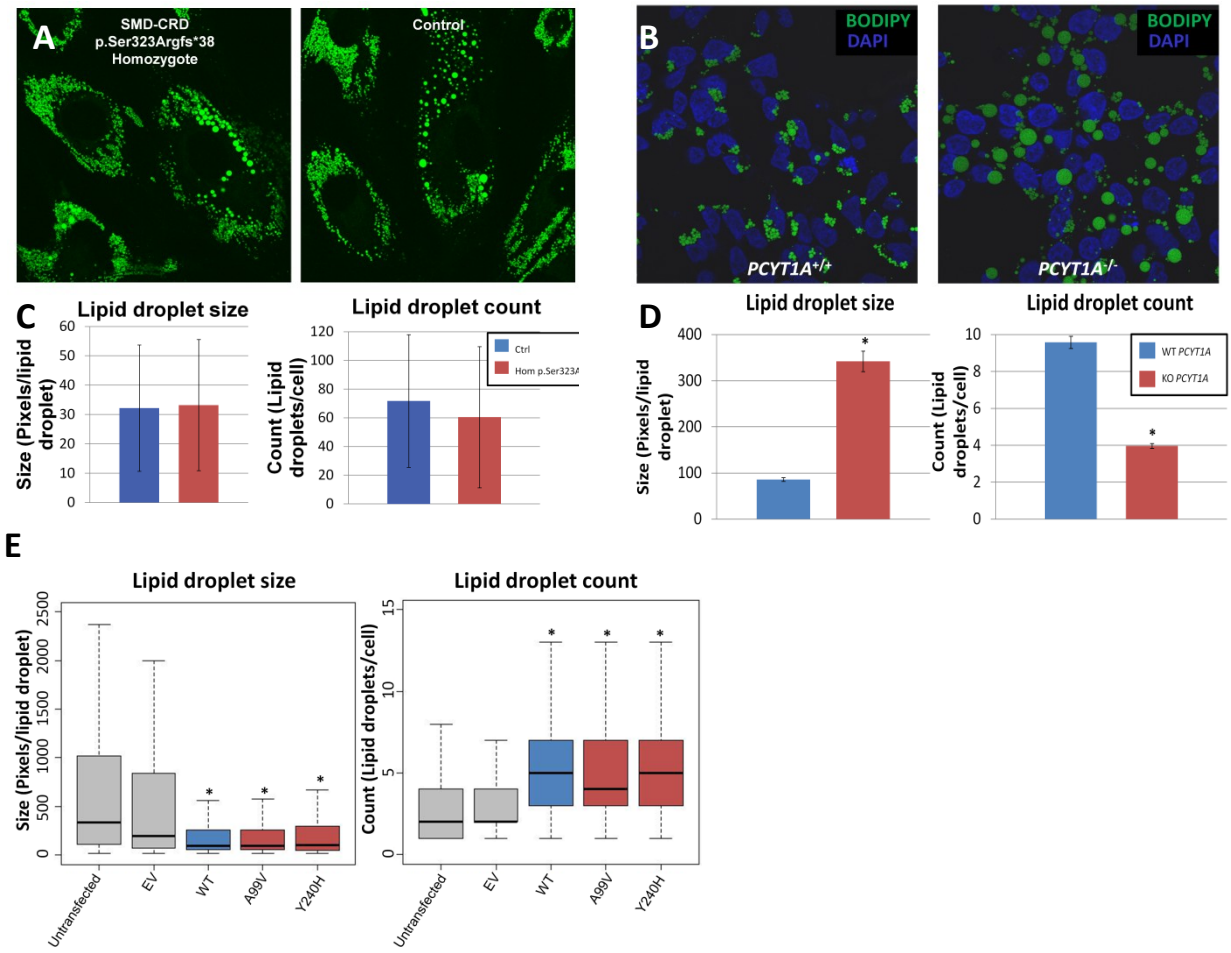


Figure 5. *PCYT1A*-null HEK293 and ATDC5 cells have normal proliferation rates. A) Wild-type and *PCYT1A*-null HEK293 cells were grown in either normal serum or delipidized serum up to 144 hours and counted every 24 hours. Growth rates were comparable for both cell lines under normal serum; however, *PCYT1A*-null HEK293 cells actually grew more rapidly in delipidized serum than their wild-type counterparts. B) Undifferentiated wild-type or *Pcyt1a*-null ATDC5 cells were grown for 5 days and counted every 24 hours using absorbance-based cell counting. At all time points, wild-type and *Pcyt1a*-null cells had comparable rates of growth.

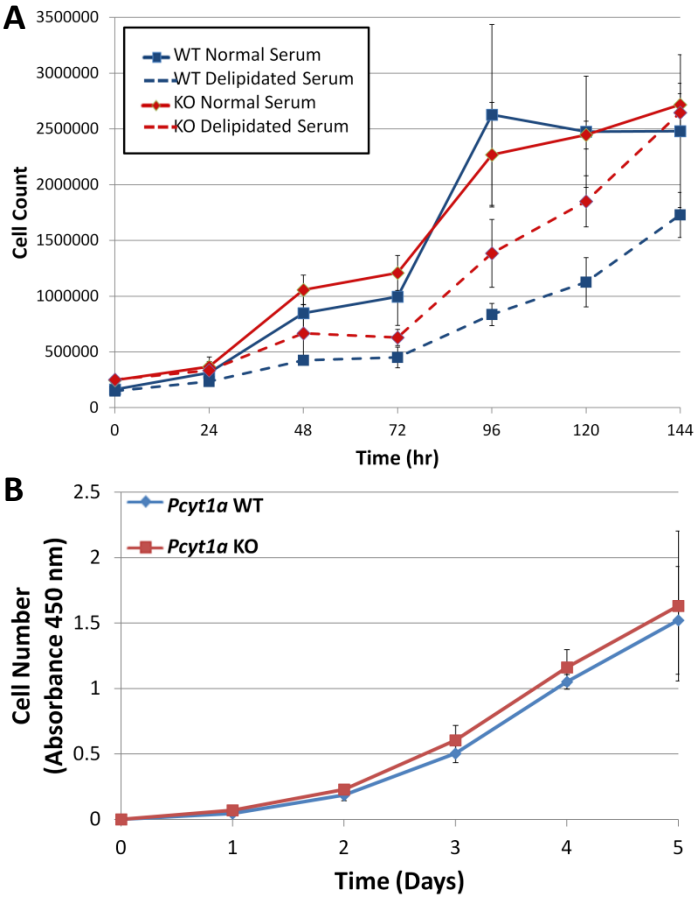


Figure 6. Involvement of Pcyt1a in ATDC5 chondrocyte differentiation. A) Western blot analysis shows that CCTα steady-state levels remain stable over the course of 21 days of chondrocyte differentiation. B) Rates of chondrocyte differentiation are increased in *Pcyt1a*-null ATDC5 cells, as measured by absorbance reading of Alcian blue staining. n=3 biological replicates for each time point.

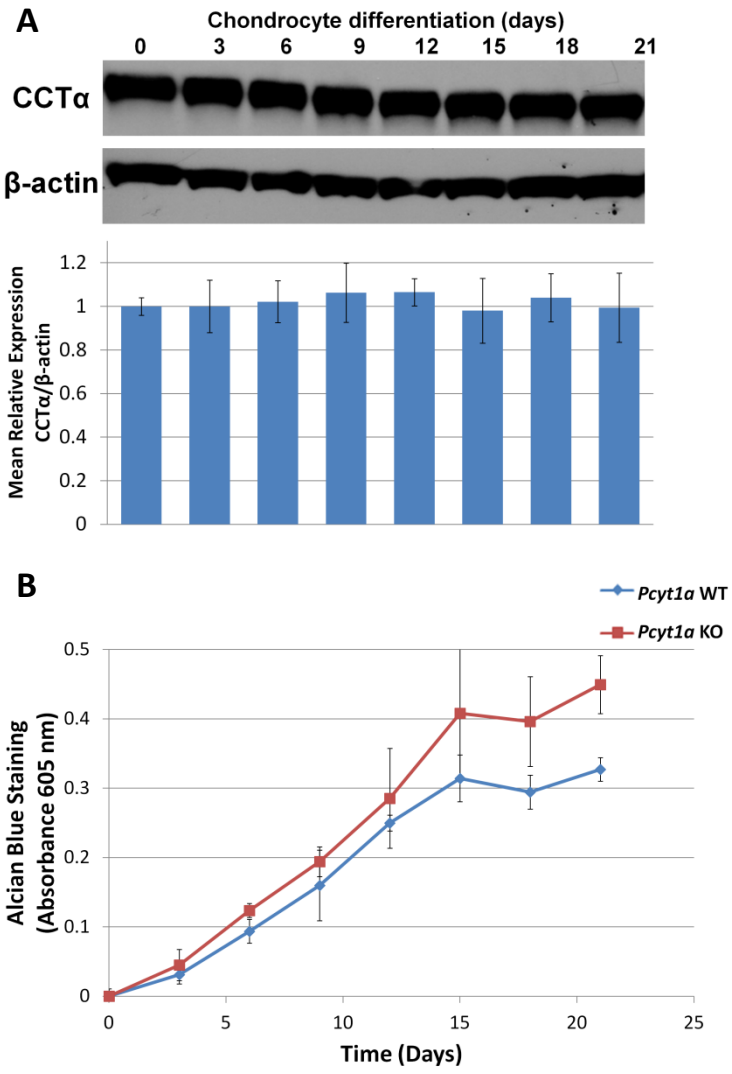
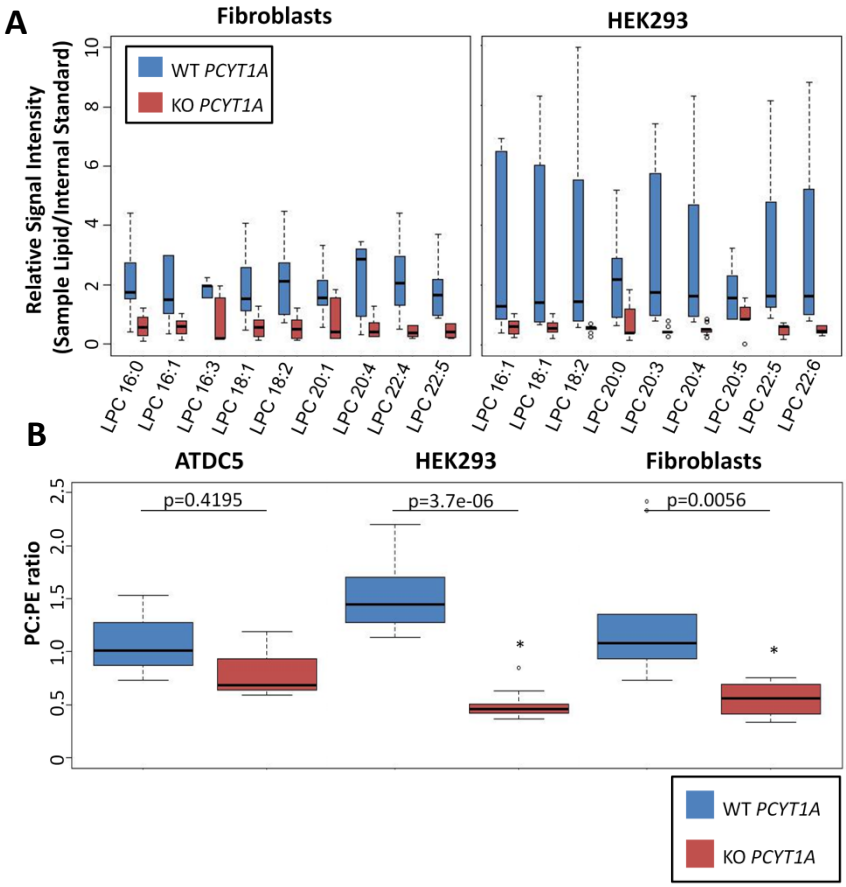


Figure 7. Untargeted lipidomics reveal decreases in LPCs and in PC:PE ratio in SMD-CRD patient fibroblasts and *PCYT1A*-null HEK293 cells. A) Several specific lysophosphatidylcholine (LPC) species (acyl chain compositions denoted) were decreased in both SMD-CRD patient fibroblasts and *PCYT1A*-null HEK293 cells as compared to wild-type controls. B) Ratios of total phosphatidylcholine (PC) to phosphatidylethanolamine (PE) were decreased in both SMD-CRD patient fibroblasts and *PCYT1A*-null HEK293 cells but not *Pcyt1a*-null ATDC5 cells as compared to wild-type controls.



Chapter 6: Conclusion

From this work, we have seen that numerous challenges exist for the detection of variants from next-generation sequencing studies as well as the functional characterization studies that follow in their wake. However, as time has progressed, novel methodological developments and analytic strategies have become available which increase our ability to identify and characterize genetic variants underlying Mendelian disorders. For instance, long-read sequencing methodologies are evolving and becoming increasingly more affordable, so we can sequence larger blocks of DNA and better detect structural variants, copy number variants, or disease haplotypes. Whole genome sequencing is also becoming more cost-effective, so as we approach saturation for variant detection in the exome, we can turn toward genome sequencing for families whose causal variants were not detectable via exome sequencing. In addition, methods for variant detection are progressing—particularly for copy number and structural variants—allowing us to revisit existing sequence data with new tools. Alternatively, we can couple existing methods such as linkage analysis with next-generation sequencing in order to solve a greater proportion of cases.

Researchers and clinicians are increasingly recognizing that families with each rare Mendelian disorder are limited, as are the resources with which to study them. Consequently, collaborative efforts are increasing, both through traditional mechanisms (see Chapter 2 for an example) and through tools such as GeneMatcher which facilitate genetic matchmaking on a larger scale (Sobreira, Schiettecatte, Valle, & Hamosh, 2015). Moreover, many groups are now integrating mRNA expression data from individual RNA-seq experiments or publicly available databases (e.g. Allen Brain Atlas and the

GTEx project) as well as proteomic studies to prioritize genes which are mutated and also have altered expression profiles (Genotype-Tissue Expression Project, www.gtexportal.org; Allen Brain Atlas, www.brain-map.org).

Large databases harboring variant frequency information (e.g. ExAC and gnomAD) as well as variant-associated phenotypic information (e.g. ClinVar) have enhanced our ability to filter variants based on their population frequencies or known clinical associations (Landrum et al., 2014; Lek et al., 2016). Many countries whose populations were previously underrepresented in genetic databases are also undertaking large-scale sequencing projects, which will enrich our understanding of human variation more globally. Moreover, our knowledge of which genes are essential for life in humans or other model organisms is also evolving through resources like the knockout mouse project and the human knockout project (Lloyd, 2011; Saleheen et al., 2017), which demonstrate the consequences of deleting specific genes. The Precision Medicine Initiative is also underway, which will elucidate relationships between genomics and phenotypic outcomes as they evolve over time (Terry, 2015).

Moreover, new computational and statistical methods are being developed that can be applied to sequencing studies. Some of these tools include improved splice site detection algorithms (M. Lee et al., 2017), catalogs of noncoding genetic elements such as those developed by the ENCODE project (Dunham et al., 2012), and tools for the spatial clustering of de novo missense variants (Lelieveld et al., 2017). In addition, we can apply more robust statistical methods such as genetic burden tests for cohort analyses (S. Lee, Abecasis, Boehnke, & Lin, 2014). Although statistical tools for detecting

oligogenic contributors to Mendelian disease have not yet been developed, such an advance is anticipated going forward.

Various methods are currently available for prioritization of candidate genes and variants, such as genic intolerance and CADD scores (Kircher et al., 2014; Petrovski et al., 2013), and additional scores have been developed for applications such as ranking variants on a genome-wide level or based on the protein domains in which they fall (Gussow, Petrovski, Wang, Allen, & Goldstein, 2016; Smedley et al., 2016; Wiel, Venselaar, Veltman, Vriend, & Gilissen, 2017).

As a more robust catalog of candidate genes and variants is developed, we will be faced with the need to characterize their functional consequences at the bench. As demonstrated in Chapter 5, this can be quite challenging and may reveal unanticipated mechanistic complexities that take time to unravel. However, several tools now exist that improve our ability to functionally characterize variants and may ease this process in the future. For instance, we can now generate knockout or knock-in models fairly quickly using CRISPR/Cas9-mediated genome editing. This method may ameliorate the challenges of allelic heterogeneity by enabling us to model specific alleles, or even to examine the functional consequences of all possible changes in a gene through saturation genome editing (Findlay, Boyle, Hause, Klein, & Shendure, 2014). Genome editing can be applied to various model systems, including cell lines, animal models, and induced pluripotent stem cells, which together give us a broadly expanded repertoire for understanding the functional consequences of genetic variation.

In the end, next-generation sequencing endeavors, increasingly sophisticated analytic strategies and functional follow-up studies will have far-reaching consequences

for patients with Mendelian disorders, their families, and society at large. We now have a wide array of disorders which we can target with pharmaceutical interventions or gene therapies in order to ameliorate phenotypic manifestations, and expect this will bolster our understanding of the biological systems and mechanisms underlying these and other disorders. Furthermore, the knowledge we gain from sequencing will enhance genetic counseling, carrier screening, and prenatal testing, and perhaps even lead to phenotypic expansion for known disorders. Our efforts in Mendelian genetics have been tremendously exciting and informative; we look forward to the prospects the future will hold.

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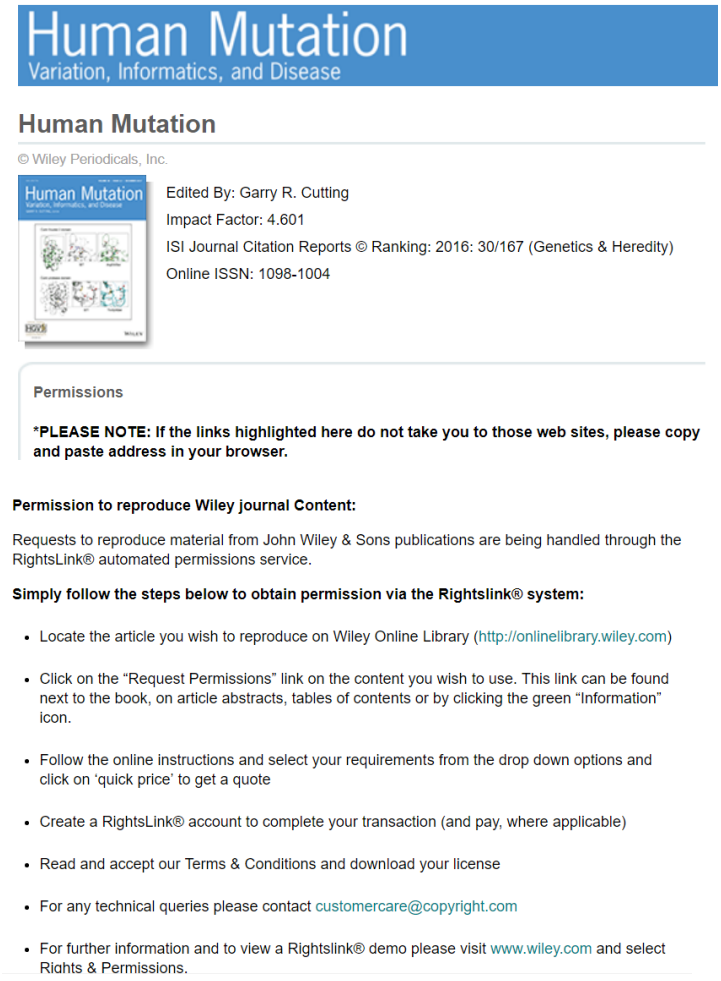

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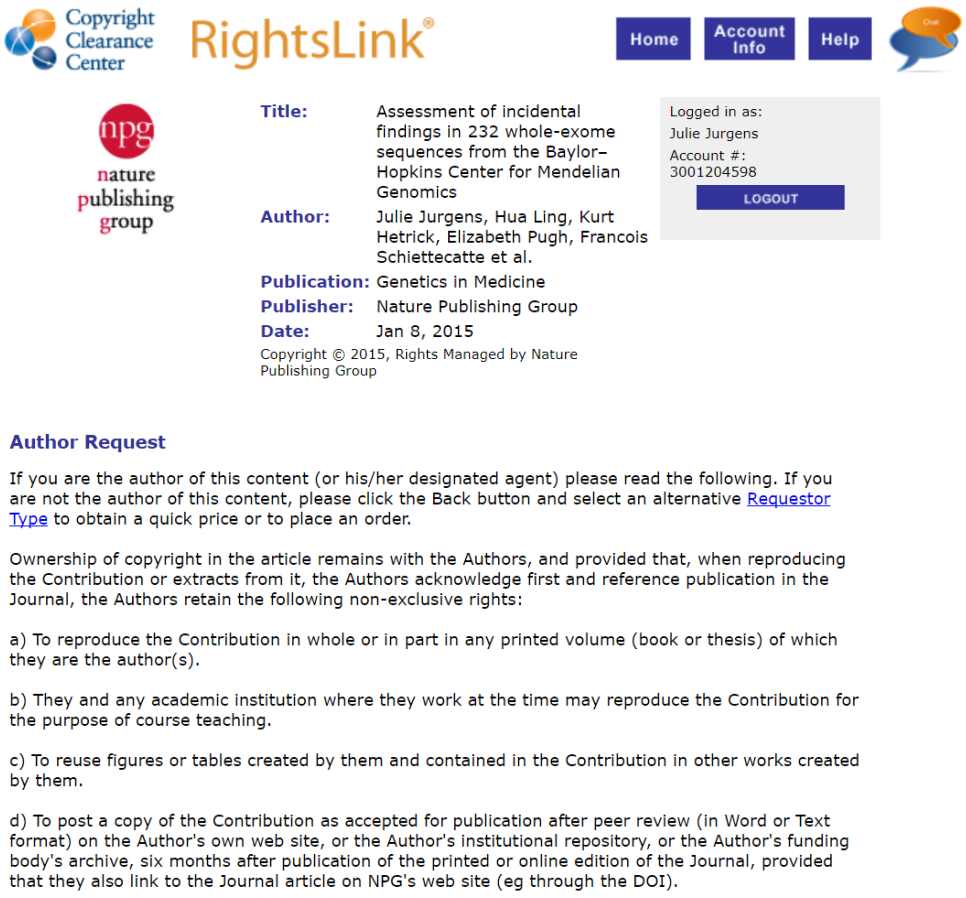

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Curriculum Vitae

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707 N. Broadway, Miller Research Building 512
Baltimore, MD 21205
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EDUCATION

Johns Hopkins University School of Medicine August 2012-Dec 2017
Baltimore, MD
Ph.D. Candidate, Predoctoral Training Program in Human Genetics

University of Illinois at Urbana-Champaign August 2006-May 2010
Champaign, IL
Bachelor of Science in Molecular and Cell Biology, Chemistry Minor
GPA: 3.62/4.00

PROFESSIONAL EXPERIENCE

Johns Hopkins School of Medicine Baltimore, MD Aug 2012-Dec 2017
Predoctoral Training Program in Human Genetics
Visual Neuroscience Training Program
Lab of David Valle, M.D.
PhD Candidate

- Developed and characterized model systems to elucidate the pathophysiological mechanisms underlying spondylometaphyseal dysplasia with cone-rod dystrophy.
- Investigated novel genetic bases of unexplained Mendelian disorders through next-generation sequencing analysis at the Baylor-Hopkins Center for Mendelian Genomics.
- Secured independent research funding to conduct a trainee-directed whole genome sequencing project in collaboration with a fellow graduate student. Won an independent fellowship to obtain financial support and pursue educational opportunities in visual neuroscience.
- Delivered written and oral presentations, including manuscripts for peer review.
- Supervised and mentored undergraduate trainees and junior graduate students.

Children's National Medical Center Washington, DC June 2010 – Feb 2012
University of Maryland School of Medicine Baltimore, MD Feb 2012-Aug 2012
Lab of Pedro Jose, Ph.D.
Research Technician

- Studied mechanisms underlying essential hypertension through a number of collaborative projects. Supervised breeding, maintenance, and genotyping of a large mouse colony and helped coordinate shipment to a new institution.
- Presented results on a weekly basis, co-authored publications, and mentored three high school students.

University of Illinois at Urbana-Champaign Champaign, IL May 2008 – May 2010
Lab of David Clayton, Ph.D.

Undergraduate Research Assistant

- Investigated modulations in gene expression associated with auditory learning in zebra finches. Completed a senior thesis which was awarded High Distinction and communicated findings verbally and orally.

University of Illinois at Urbana-Champaign Champaign, IL Sept 2009 – May 2010
Lab of Rebecca Krisher, Ph.D.

Undergraduate Laboratory Assistant

- Studied metabolic and reproductive features of Ossabaw pigs in relation to Polycystic Ovarian Syndrome (PCOS).
- Worked with a team to perform ultrasounds, obtain body measurements, collect blood and tissue samples, and care for animals.

PUBLICATIONS

Florwick A, Dharmaraj T, **Jurgens J**, Valle D, Wilson KL. (2017). *LMNA* sequences of 60,706 individuals link the p.G602S variant to Type 2 Diabetes and reveal novel missense variants shared by lamin A and C, or unique to Prelamin A. *Front Genet*. 8: 79.

Schossig A, Bloch-Zupan A, Lussi A, Wolf NI, Raskin S, Cohen M, Giuliano F, **Jurgens J**, Krabichler B, Koolen DA, Sobreira NL, Maurer E, Muller-Bolla M, Penzien J, Zschocke J, Kapferer-Seebacher I. (2017). *SLC13A5* is the second gene associated with Kohlschütter-Tönz syndrome. *J Med Genet*. 54(1):54-62.

You J, Sobreira NL, Grange DK, Gable D, **Jurgens J**, Belnap N, Siniard A, Szelinger S, Schrauwen I, Richholt RF, Vallee SE, Palko Dinulos MB, Valle D, Armanios M, Hoover-Fong J. (2016). A Newly Recognized Intellectual Disability Disorder Caused by Variants in *TELO2,* a gene encoding a component of the TTT complex. *Am J Hum Genet*. 98(5): 909-918.

Jurgens J, Sobreira N, Modaff P, Reiser CA, Seo SH, Seong MW, Park SS, Kim OH, Cho TJ, Pauli RM. (2015). Novel *COL2A1* variant (c.619G>A, p.Gly207Arg) manifesting as a phenotype similar to progressive pseudorheumatoid dysplasia and spondyloepiphyseal dysplasia, Stanescu type. *Hum Mutat.* 36(10): 1004-8.

Jurgens J, Ling H, Hetrick K, Pugh E, Schiettecatte F, Doheny K, Hamosh A, Avramopoulos D, Valle D, Sobreira N. (2015). Assessment of incidental findings in 232 whole exome sequences from the Baylor-Hopkins Center for Mendelian Genomics. *Genet Med*. 17(10): 782-8.

Minillo RM, Sobreira N, de Faria Soares Mde F, **Jurgens J**, Ling H, Hetrick KN, Doheny KF, Valle D, Brunoni D, Perez AB. (2014). Novel Deletion of SERPINF1 Causes Autosomal Recessive Osteogenesis Imperfecta Type VI in Two Brazilian Families. *Mol Syndromol*. 5(6):268-75.

Ennis RC, Asico LD, Armando I, Yang J, Feranil JB, **Jurgens JA**, Escano CS Jr, Yu P, Wang X, Sibley DR, Jose PA, Villar VA. (2014). Dopamine D1-like receptors regulate the α1A adrenergic receptor in human renal proximal tubule cells and D1-like dopamine receptor knockout mice. *Am J Physiol Renal Physiol.* 307(11): F1238-48.

Hoover-Fong J, Sobreira N, **Jurgens J**, Modaff P, Blout C, Moser A, Kim O-H, Cho T-J, Cho SY, Kim SJ, Jin D-K, Kitoh H, Park W-Y, Ling H, Hetrick KN, Doheny KF, Valle D, Pauli RM. (2014). Mutations in *PCYT1A*, encoding a key regulator of phosphatidylcholine metabolism, cause spondylometaphyseal dysplasia with cone-rod dystrophy. *Am J Hum Genet*. 94(1): 105-12.

Villar VA, Armando I, Sanada H, Frazer LC, Russo CM, Notario PM, Lee H, Comisky L, Russell HA, Yang Y, **Jurgens JA**, Jose PA, Jones JE. (2013). Novel role of sorting nexin 5 in renal D(1) dopamine receptor trafficking and function: implications for hypertension. *FASEB J*. 27(5):1808-19.

Jones JE, **Jurgens JA**, Evans SA, Ennis RC, Villar VA, Jose PA. (2012). Mechanisms of fetal programming in hypertension. *Int J Pediatr*. 2012:584831.

LEADERSHIP AND VOLUNTEER EXPERIENCE

American Society of Human Genetics (ASHG) Bethesda, MD Jan 2015-Dec 2017
Training and Development Committee

- Work with a team of ASHG trainees and staff to develop events and resources for trainees.
- Conceptualized and generated a quarterly newsletter for ASHG trainees. Lead a working group in which I contribute and edit original articles, manage a group of trainee writers, and finalize content with ASHG staff.
- Developed an initiative to make informational videos about ASHG resources. Led working groups in 2015 and 2016 to script, narrate, film, and edit videos.
- Plan career development events for annual ASHG meetings. Invite speakers, coordinate session logistics, and moderate career development sessions.
- Review annual trainee applications for all ASHG committees.

Predoctoral Training Program in Human Genetics Jan 2015-Jan 2017

Johns Hopkins University
Student Representative

- Plan and execute annual student orientation and recruitment events.
- Chaired a committee of students for selection and invitation of a speaker for the 2015 Barton Childs Memorial Lectureship. Coordinated lecture logistics and introduced the speaker.
- Helped to organize and co-moderate the 2017 Human Genetics Career Day.
- Collaborate with program administrators to develop new program initiatives, deliver information to the student body, and collect feedback to improve departmental resources and events.

Online Mendelian Inheritance in Man (OMIM) October 2015-June 2016
Johns Hopkins University
Volunteer Article Writer

- Contributed regular articles to OMIM describing genes and their relation to human phenotypes.

American Society of Human Genetics, 2016 Annual Meeting October 2016
Co-moderator, "Diseases of the Nervous System" Platform Session

- With a fellow trainee, managed session timing, speaker introductions, and question/answer period. Coordinated logistics with presenters and ASHG's Program Committee prior to the meeting.

Leadership Alliance National Symposium 2016 July 2016
Session Moderator and Poster Presentation Judge

- Reviewed and provided feedback on abstracts, posters, and oral presentations for undergraduate symposium attendees.
- Moderated a session for undergraduate presenters. Coordinated logistics prior to the meeting and managed introductions, timing, and question/answer period.

Molecular and Cell Biology Advising University of Illinois Oct 2008–May 2010
Undergraduate Research Information Workshop Leader

- Advised 10-20 undergraduates per month on how to become involved in campus research.
- Led group discussions, delivered presentations, and collaborated with others to develop departmental events.

Future Medical Research Investigators University of Illinois Aug 2008 – May 2010
Treasurer, August 2009-May 2010

- Informed students of the dual MD/PhD degree option, how to pursue it, and how to use available resources to learn more about programs.
- Invited university affiliates and external guest speakers to deliver presentations.
- Managed accounts and delivered presentations to the student funding board to raise finances.

Illini Medical Screening Society University of Illinois Jan 2009 – May 2010
General Member

- Provided free health education and screenings to local low-income or uninsured individuals.
- Measured blood pressure and metabolites to determine participants' likelihood of developing hypertension, diabetes, and hyperlipidemia.
- Helped to organize screenings and recruit local doctors to interpret test results and guide participants toward additional resources.

AWARDS AND HONORS

Schepens Eye Research Institute, Massachusetts Eye and Ear Hospital, Harvard Medical School

2018 Molecular Bases of Eye Diseases Postdoctoral Fellowship Recipient

Burroughs-Wellcome Fund/ Maryland Genetics, Epidemiology, and Medicine Program

2016 Wolfe Street Competition Award Co-Recipient for proposal to detect novel genetic mechanisms of strabismus through whole genome sequencing
The Wolfe Street Competition provides $15,000 for an independent research project led by two trainees from the Johns Hopkins Bloomberg School of Public Health and the Johns Hopkins University School of Medicine to foster collaboration between laboratory and population scientists.

Johns Hopkins University Wilmer Eye Institute and Department of Neuroscience

2015 Visual Neuroscience Training Program Graduate Fellowship Recipient

American Society of Human Genetics

2013 ASHG/ Charles J. Epstein Trainee Award for Excellence in Human Genetics Research, Semifinalist

University of Illinois at Urbana-Champaign

2010 Cell & Developmental Biology Department Award for Distinguished Research-Best Senior Thesis
2010 Graduation with High Distinction

TEACHING AND MENTORSHIP

Graduate Course in Advanced Topics in Human Genetics Nov 2014-May 2015
Johns Hopkins University
Teaching Assistant

- Worked with course instructors and a fellow graduate student to shape course content, manage the course, and answer students' questions.
- Led weekly discussion sections, wrote and graded assignments and exams, developed review materials, and coordinated course logistics.

Predoctoral Training Program in Human Genetics Sept 2015-Dec 2017
Johns Hopkins University

Graduate and undergraduate student mentor

- Mentor undergraduate and junior graduate students in their laboratory rotations and thesis projects.
- Develop projects for students and supervise progress in lab.
- Help students design, conduct, and troubleshoot experiments. Assist them in developing abstracts, posters, and oral presentations. Provide ongoing resources for scientific and career development.

Thomas Jefferson High School for Science and Technology June 2010-Aug 2012
McLean, Virginia
High school student mentor

- Taught high school students techniques in molecular biology, supervised laboratory progress, and offered troubleshooting assistance.
- Helped students develop written and oral presentations, write scientifically, edit college applications, and navigate educational and career development.

Mentees:

High school students: Alan Barte, Rishav Adhikari, Riley Ennis
Undergraduate students: Adam Uppendahl, Sophie Nguyen
Graduate students: Arianna Franca, Sarah Robbins, Anh-Thu Lam

CONFERENCE PRESENTATIONS

Assessment of incidental findings in whole exome sequencing data from the Baylor-Hopkins Center for Mendelian Genomics. Platform Presentation. American Society of Human Genetics 2013.

Abnormal phospholipid metabolism due to variants in *PCYT1A* causes spondylometaphyseal dysplasia with cone-rod dystrophy. Poster Presentation. Hopkins MD-GEM Genetics Research Day 2014.

Assessment of incidental findings in whole exome sequencing data from the Baylor-Hopkins Center for Mendelian Genomics. Poster Presentation. Hopkins MD-GEM Genetics Research Day 2014.

Abnormal phospholipid metabolism due to variants in *PCYT1A* causes spondylometaphyseal dysplasia with cone-rod dystrophy. Poster Presentation. American Society of Human Genetics 2014.

Strategy for Generating and Characterizing a Zebrafish Knockout Model of Spondylometaphyseal Dysplasia with Cone-rod Dystrophy. Poster Presentation. Hopkins MD-GEM Genetics Research Day 2015.

Strategy for Generating and Characterizing a Zebrafish Knockout Model of Spondylometaphyseal Dysplasia with Cone-rod Dystrophy. Poster Presentation. American Society of Human Genetics 2015.

Phenotypic characterization of cell lines from patients with spondylometaphyseal dysplasia with cone-rod dystrophy (SMD-CRD). Poster Presentation. American Society of Human Genetics 2016.

Investigation of novel genetic bases for strabismus using linkage analysis and next-generation sequencing. Hopkins MD-GEM Genetics Research Day 2017.

Investigation of novel genetic bases for strabismus using linkage analysis and next-generation sequencing. Poster Presentation. American Society of Human Genetics 2017.

PROFESSIONAL SOCIETY MEMBERSHIPS

Member, American Society of Human Genetics June 2013-Present